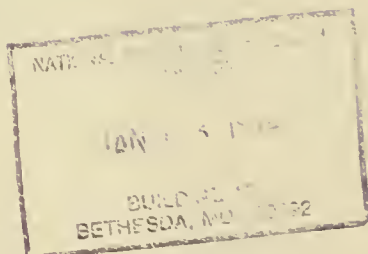






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**ANNUAL REPORT OF THE SCIENTIFIC DIRECTOR
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
OCTOBER 1, 1992 - SEPTEMBER 30, 1993**

The Division of Intramural Research consists of 18 different units (17 Laboratories and Branches and one independent Section) working on a wide range of projects. The total staff numbers about 550 of whom about 65 are permanent, doctoral-level scientists, 225 are post-doctoral fellows and the remainder technical and administrative support staff. Approximately 150 guest workers contribute importantly to the scientific research. The annual budget is about \$100,000,000, of which one-third supports all laboratory research expenses, one-third the clinical research and one-third salaries and benefits of the staff. The research is conducted in laboratories totalling about 130,000 square feet and 80 research beds in the Clinical Center. None of these figures has changed significantly for several years.

The following is a brief summary of some of the research conducted during this last year. These incomplete highlights touch on only a small fraction of a large and diverse research program. The summaries provided under the individual headings for each Laboratory and Branch should be read to acquire a better understanding of the breadth and quality of the scientific interests and accomplishments of the Division of Intramural Research.

Research in the **Pulmonary Branch** focused on two areas: 1) gene therapy, including *in vivo* gene transfer strategies for the lung and other organs, and 2) inflammatory processes in the lung. In the area of gene therapy, they concentrated on cystic fibrosis, an autosomal recessive disease that results from a well characterized mutation of the cystic fibrosis transmembrane regulator (CFTR) gene. They showed that the normal CFTR gene can be directly transferred to airway epithelial cells in cotton rats by means of a replication deficient recombinant adenovirus (Ad) vector. Cells normally limit CFTR function and therefore, CFTR overexpression during *in vivo* gene therapy should have no untoward effects. The basic anatomy of the lung, i.e., anastomoses between pulmonary and bronchial circulations, may enable targeting of all categories of cells necessary for gene therapy for cystic fibrosis to succeed using an adenovirus vector. They confirmed that expression of the CFTR gene could be down-regulated at the transcriptional level by inflammatory stimuli. This is a potential problem for gene therapy since individuals with cystic fibrosis generally have long-standing pulmonary infections. *In vitro* studies suggest that constitutive viral promoters will be resistant to inflammation-induced down-regulation, an important factor in the design of vectors to be used for the correction of cystic fibrosis by gene transfer. They are also interested in *in vivo* gene therapy for alpha 1 antitrypsin (alpha 1 AT) deficiency, again by means of an adenovirus vector. They demonstrated the feasibility of adenovirus-mediated gene transfer targeted to the peritoneal mesothelium as a site for the synthesis and secretion of normal human alpha 1 AT. In the area of

pulmonary inflammatory diseases, they showed the importance of oxidant-mediated injury and that H_2O_2 plays a central role in this epithelial injury. They were able *in vitro* to transfer catalase cDNA to lung cells, and show the feasibility of adenovirus mediated transfer of catalase cDNA to human bronchial epithelium as a way of protecting against oxidant injury.

The **Section on Pulmonary and Molecular Immunology** continued its studies of the structure and function of the IL-2 receptor. This year they showed that mutation of IL-2R γ results in x-linked severe combined immunodeficiency (XSCID) in humans. This resulted from the group's observation that the IL-2R γ gene is located on the X chromosome in a position previously determined to be the location for XSCID. A study of transformed cell lines derived from three XSCID patients showed that each of the three unrelated patients had a different point of mutation, resulting in a different premature stop codon. They also showed that IL-2 deficient mice and human SCID patients deficient in IL-2 production have normal levels of T-cells. This suggests that the IL-2-2R γ is a component of more than one cytokine receptor system, a hypothesis that this group recently confirmed. In order to prepare a murine model, they cloned and sequenced the gene for murine IL-2 R γ .

The **Cardiology Branch** continues its research in hypertrophic cardiomyopathy (HCM). They showed in more than 150 patients that dual chamber pacing is effective in relieving drug-refractory symptoms and in reducing LV outflow obstruction. This has rendered traditional therapy with either a LV myectomy or mitral valve replacement unnecessary. They found that chronic pacing alters electrical and hemodynamic cardiac properties that persist even after the pacing is discontinued. Further studies are underway to determine the molecular basis of this observation. In studies of endothelial dysfunction, they showed that patients with hypertension or hypercholesterolemia have impaired endothelium-dependent vascular responses to acetylcholine, and that this abnormality is related to a defect in the nitric oxide system. Now they have found that this impaired endothelial function is not related to decreased availability of the precursor, L-arginine. They showed that reinjection of thallium, after a 3-4 hour period of redistribution identifies patients with viable but dysfunctional myocardium. The results of the thallium reinjection study are essentially the same as those from positron emission tomography, as far as predicting the recovery of function after revascularization procedures. This is important information since PET is very expensive and requires a cyclotron, while thallium imaging is readily available at most hospitals equipped for routine nuclear medicine procedures. In their study of molecular-based therapeutic strategies, they demonstrated that an adenovirus-delivered gene is capable of inhibiting smooth muscle cell proliferation *in vitro*. Further work is underway to see if such gene therapy might prevent restenosis after percutaneous coronary angioplasty.

The **Laboratory of Cardiac Energetics** has concentrated on the complex interaction between energy conversion processes in the heart, muscle contraction and coronary blood flow. They recently showed that one or more elements of the complex oxidative phosphorylation process are up-regulated by an increase in NADH. They found a linear relationship between NADH-stimulated Vmax and FI-ATPase hydrolysis activity. Thus, they hypothesized that the inner mitochondrial membrane is the intermediary between NADH and FI-ATPase activity. Using a recently developed assay technique for the dog heart *in vivo*, they demonstrated that the regulation of FI-ATPase activity may play a key role in the short-term regulation of oxidative phosphorylation.

Research in the **Laboratory of Molecular Cardiology** complements research on hypertrophic cardiomyopathy in the Cardiology Branch. Thus, they showed that the mutant b-myosin heavy chain (MHC) gene is expressed together with the wild-type myosin in both skeletal and cardiac muscles of patients with specific mutations in the b-MHC gene. They showed that myosin isolated from both the cardiac and skeletal muscle of HCM patients propels actin filaments at a significantly lower velocity than myosin isolated from normal control muscles. The most dramatic decreases in the rate of translocation were correlated with the mutated amino-acid being closest to the ATP binding domain (Cys 162) and near to the actin binding domain (Gln 403).

The **Pathology Branch** made use of Sirius red staining and video densitometry to determine the concentration of total, interstitial and perivascular collagen in sections of the ventricular septum from 16 patients with HCM. The quantity of total collagen was increased 4-fold and that of interstitial collagen 7-fold in patients with HCM compared to normal controls. There was no correlation between the age of the patient, the ventricular septal thickness, or the degree of myofiber disarray, and the quantity of collagen in the patients with HCM. This indicates that many factors can induce myocardial fibrosis in patients with HCM. They also demonstrated that endomyocardial biopsy from the right side of the ventricular septum is an important and accurate method of evaluating the degree of cardiac toxicity produced by the administration of anti-neoplastic agents of the anthracycline family.

Researchers in the **Clinical Hematology Branch** have also collaborated in the identification of the genetic abnormality in HCM. The MHC gene is abnormal in 10-30% of affected kindreds with HCM. Thirteen distinct mutations in the head or head-rod junction region of the myosin molecule have been identified in 17 different kindreds. Presymptomatic diagnosis can now be accomplished in these families and it is possible to correlate the presence of certain mutations with high penetrance, early onset of the disease, and a high frequency of sudden death. Thus, important genetic data are now available to help determine the prognosis of affected individuals. The Clinical Hematology Branch developed a murine model in which they demonstrated the ability of certain cytokine combinations to enhance gene transfer into murine stem cells *in vitro*. They purified murine stem cells more

than a thousandfold, and are able to reconstitute individual animals with as few as 100 of these stem cells. The insights acquired from this murine model were applied to an autologous bone marrow transplantation model in nonhuman primates. Using positive immunoselection for cells co-expressing the CD34 and the THY 1 antigens, they achieved reconstitution of irradiated recipients with as few as 5×10^5 cells/kg. Meanwhile, they began a program of autologous bone marrow transplantation for patients with either multiple myeloma or chronic myelogenous leukemia. So far, six patients have received peripheral blood and bone marrow cells transduced with retroviral vector preparations. Genetically modified cells have been detected in the peripheral blood during the early stages of post-transplantation.

The **Hematology Branch** found a probable explanation for the extraordinary erythroid tropism of B19 parvovirus by identification of its cellular receptor. This receptor is globoside or P antigen on red blood cells. In rare individuals who genetically lack the P antigen, their marrow cells in tissue culture were not susceptible to parvovirus infection. They found that intensive immunosuppression with antithymocyte globulin, cyclosporin, and methylprednisolone is extremely effective in patients with severe aplastic anemia, resulting in 70% hematologic remission rates at one year post therapy. This is clearly a dramatic improvement over the results in such patients with ATG alone.

The **Molecular Hematology Branch** has successfully constructed retroviral vectors that express SCD4, transdominant REV mutants, antisense RNA for TAR, and HIV inducible vectors for α -interferon, cytosine deaminase and diphtheria toxin. The development of these retroviral vectors is necessary for further progress in the use of gene therapy to treat various disorders.

Research in the **Molecular Disease Branch** focused on the genetic expression of apoB, the major structural apolipoprotein on chylomicrons and VLDL. They found that when intestinal cells in culture were transfected with a plasmid encoding the thyroid nuclear receptor and then treated with thyroid hormone, there was a 6-fold increase in the expression of the apoB mRNA editing process compared to control cells. These studies provide the first insight into the molecular mechanism whereby thyroid hormone modulates apoB gene expression and the apoB mRNA editing process. Their studies also showed that lipoprotein lipase has a loop of 22 amino-acids (cysteine 216 to cysteine 239) that contains two amphipathic helices and is essential for the hydrolysis of emulsified, long chain fatty acid triglycerides. There is evidence that this structure is important in modulating the access of lipid substrates to the catalytic site of the enzyme. They found a patient who has hepatic lipase deficiency with an established molecular defect in the hepatic lipase gene. This patient presented with a pseudo Type III hyperlipoproteinemia, including hypertriglyceridemia, pancreatitis, and increased plasma beta VLDL, as well as HDL II and early heart disease. This patient shows the normal gene sequence necessary for splicing of hepatic lipase mRNA and highlights the fact that alternate splicing is a novel mechanism leading to an hepatic enzyme deficiency.

The **Hypertension-Endocrine Branch**, using a murine model for high output congestive heart failure, showed that activation of the renin-angiotensin-aldosterone system (RAAS) is the major reason such animals in congestive heart failure do not respond to either endogenous or exogenous atrial natriuretic factor (ANF). When the RAAS is blocked by either losartan, a potent inhibitor of angiotensin II receptors, or by an angiotensin-converting enzyme inhibitor (ACEI), the animals compensate, i.e., their urinary output of sodium returns to normal levels, their edema disappears, and they survive. They showed that neutral endopeptidase EC.3.4.24.11 is not the enzyme that converts Big endothelin (BET) to endothelin (ET), that endothelium is not required for the vascular effects of either ET-1 or BET-1, that endothelium is not required for the conversion of BET to ET, and therefore that the endothelin converting enzyme (ECE) must exist in vascular smooth muscle cells. Using an *in vivo* murine model, they demonstrated two different phenotypes for drug-induced cardiac hypertrophy (CH), one due to chronic α -adrenergic stimulation with phenylephrine (PE), and the other due to chronic β -adrenergic stimulation with isoproterenol (ISO). They characterized these different phenotypes and showed that they differ in the rates of induction and reversal of CH; the susceptibility of populations to the development of CH according to age and gender; the percentage of animals that develop CH; and the biochemical and functional changes that accompany CH. They also showed that the stimuli for the initiation and maintenance of CH are different. Thus, ISO treatment initiates CH, while angiotensin II maintains CH. They are now in the process of evaluating the clones produced by subtractive hybridization that appear responsible for the differential expression of these two phenotypes.

The **Laboratory of Kidney and Electrolyte Metabolism** continues its studies on the mechanism of transport regulation in the inner medullary collecting duct (IMCD). They found that vasopressin treatment of an animal or fluid restriction is associated with a marked increase in intramembrane particles in the apical membrane of IMCD cells. These particles are thought to contain vasopressin-regulated water channels and, indeed, immunocytochemistry has shown an increase in the total number of water channels in IMCD cells in response to water restriction of the whole animal. They also made use of mathematical modeling to assess the mechanisms of vasopressin-mediated regulation of water permeability. When experimental data are studied with computer simulations, they are able to fit the data only when it is assumed that both endo- and exocytosis are regulated by vasopressin. Sophisticated computer modeling was also used to demonstrate that the measured permeability properties in the descending limb of the chinchilla kidney are inconsistent with a purely passive process for concentration in the inner medulla of the kidney.

Research in the **Laboratory of Biochemical Genetics** defined more clearly the role of the NK-2 homeobox gene. Thus, they showed that the NK-2H gene has a novel homeodomain secondary structure and that the NK-2 gene receives and integrates information from the ventral-dorsal and anterior-posterior gradients of gene regulators needed to generate a pattern of clusters of neuroectodermal cells that

synthesize NK-2 RNA which are precursors of different types of neuroblasts. This serves as the basis for the development of the equivalent of the spinal cord in *Drosophila*. They sequenced approximately 8 kb of a novel mouse homeobox gene, NKx-1, a homolog of the *Drosophila* NK-1 homeobox gene. They demonstrated an amazing degree of homology in a critical area in the two genes and showed that the NK-1 gene is expressed in discrete regions of the developing mouse embryo midbrain, hindbrain, spinal cord, vertebrae, and ribs. They are also studying the mechanisms related to cellular self-destruction, termed "apoptosis" or programmed cell death, by studying the genes activated by irradiation of nerve cells in vitro. They found that a variety of transcripts are increased moderately during radiation-induced neuroblast cell death. They are currently seeking even more strongly activated genes which may function in triggering programmed cell death.

The **Laboratory of Biochemistry** showed that tryptophan residues are among the most sensitive targets for destruction by ozone and that the degradation of tryptophan by ozone leads to the formation of N-formylkynurenine, kynurenine, and an as yet unidentified product. They isolated the lipofuscin, which accumulates in cells with increasing age of the animal, and demonstrated that this material is retinyl palmitate. They found that the concentration of this material in rat liver increased linearly throughout the lifetime of the animal. In studies of cultured bovine endothelial cells, they showed that transient exposure to hydrogen peroxide leads rapidly to modification of certain cellular proteins. Later, there is a drop in cellular energy, presumably as a sequence of oxidation of structural and catalytic proteins.

The **Laboratory of Biophysical Chemistry** recently acquired an electrospray triple quadrupole mass spectrometer, as well as a 600 Mhz nuclear magnetic resonance spectrometer, and has used these new tools in several areas of research. They completed about 75% of the 3-D structure of the 77 amino-acid homeodomain encoded from the homeobox gene of *Drosophila*. They found that the secondary structure appears to utilize a helix-turn-beta-pleated sheath to bind to the DNA and this represents an entirely new binding motif for homeodomain proteins. They made use of three new techniques for the separation of closely related substances, i.e., pH-zone countercurrent chromatography, cross axis centrifugation, and coil planet centrifugation. Each of these techniques proved to be uniquely useful for the separation of closely related chemical substances or even intact cells.

Research in the **Laboratory of Cell Biology** emphasized biologic functions of the myosin I family from *Acanthamoeba*. They found a specific myosin IC antibody that inhibits phosphorylation of myosin IC by kinase and inhibits actin-activated MG + 2-ATPase activity of myosin IC in vitro. When this activity was introduced into viable cells that were then exposed to hypotonic media, the cells swelled and lysed because contractile vacuole formation was arrested. This is the first evidence of a specific role for a membrane-associated myosin I. They also showed

that muscle and amoeba F-actin behave differently when added to amoeba extracts under polymerizing conditions. There appear to be stabilizing and destabilizing proteins that interact differently with the two actins. This raises serious questions about the common use of muscle F-actin as an indicator of the behavior of cell actin in non-muscle cells. In another area, they studied the fluorescence of proteins and peptides and found that contrary to accepted dogma for all fluorescent systems, the lifetime and quantum yield of tryptophan fluorescence are not directly proportional for the more than 20 proteins that they studied.

Research in the **Laboratory of Cellular Metabolism** focused on the 20-kDa ADP-ribosylation factors (ARFs) that are involved in intracellular vesicular transport to Golgi membranes. They showed that ARFs enhance all cholera toxin catalytic activities independent of the ADP ribose acceptor. From their work, it seems likely that functional specificity is achieved, at least in part, by regulation of expression of individual ARF genes in different cells or at different times during development.

Research in the **Laboratory of Chemical Pharmacology** focused on mechanisms of activation of mast cells. They identified and cloned an adenosine A-3 receptor and showed that it is also present in other types of mast cells, as well as T cells and endothelial cells. The finding that the mast cells lack either of the classic adenosine receptors clarifies paradoxical reports in the literature, for example, the inefficacy of methylxanthines in blocking the actions of adenosine and the insignificant effects of adenosine on cyclic AMP levels in tissue mast cells. They showed that H_2O_2 can cause covalent binding of the heme enzyme to protein. The formation of superoxide and H_2O_2 by the altered myoglobin raises the possibility that the alteration could lead to cellular death. In fact, they showed that incorporation of altered myoglobin into fibroblasts leads to cell death, whereas incorporation of native myoglobin does not. They also showed that some of the various isoforms of hepatic cytochrome P-450 are more sensitive to the impairing effects of nitric oxide than others. This may be due to the fact that nitric oxide may inhibit some isoforms reversibly, and others irreversibly.

ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMICAL GENETICS
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1992 through September 30, 1993

NK-2 Homeobox Gene. The first known step in the zygotic development of part of the Drosophila CNS is the expression of the NK-2 gene. During the past year, proteins that regulate the expression of the NK-2 gene were identified by determining the expression of the NK-2 gene in embryos with mutations in various genes. The results show that the NK-2 gene is activated in the ventral half of the embryo, presumably by dorsal protein, which is distributed in nuclei in a ventral-dorsal concentration gradient. The NK-2 gene is activated but not expressed in the most ventral horizontal stripe of nuclei, the mesodermal anlage, due to repression by snail, a zinc finger protein, or in the adjacent horizontal stripe of nuclei, the mesectodermal anlage, due to repression by single-minded and Enhancer of split m8, which are basic, helix-loop-helix proteins. However, the NK-2 gene is expressed by nuclei in the ventral half of the ventrolateral neurogenic anlage early in Drosophila embryonic development as the nuclei undergo commitment to the neuroblast pathway of differentiation, or soon thereafter. Initially, the NK-2 gene is expressed fairly uniformly in a horizontal stripe of nuclei about 7 nuclei in width on each side that extends over 90% of the length of the embryo. During gastrulation, the horizontal stripe of cells expressing NK-2 is converted to 12 vertical stripes by repression of the NK-2 gene in some cells. Later, 26 clusters of cells that express the NK-2 gene are formed on each side, presumably by repression of the NK-2 gene in additional cells. Therefore, 2 clusters of neuroectodermal cells that synthesize NK-2 RNA are formed per hemisegment that are precursors of many neuroblasts in the ventral nerve cord.

Twenty high-affinity and 13 low-affinity NK-2 binding sites were found in 2.2 kb of DNA from the 5'-upstream region of the NK-2 gene, which suggests that NK-2 protein may be required to maintain the expression of the NK-2 gene. Putative sites for other proteins that overlap or are adjacent to the NK-2 protein binding sites were found. The conversion of neuroectodermal cells to neuroblasts is accompanied by activation of the snail gene in the neuroblasts, thereby repressing activation of the NK-2 gene by dorsal protein. The results suggest that the NK-2 gene receives and integrates information from the ventral-dorsal and anterior-posterior gradients of gene regulators that is needed to generate a pattern of clusters of neuroectodermal cells that synthesize NK-2 RNA that are precursors of different types of neuroblasts.

One of the major goals in neurobiology is to understand how the nervous system is assembled. Studies on the NK-2 homeobox gene led to some novel ideas and to a hypothesis which predicts the overall strategy of the gene program (that is the rules) for the early development of part of the CNS of Drosophila. Every aspect of the hypothesis can be tested experimentally using the NK-2 gene. With a slight modification the hypothesis also applies to the assembly of

part of the mammalian CNS.

Circular dichroism measurements and 1D NMR spectra showed that the t_m for denaturation of the NK-2 homeodomain, NK-2H, is approximately 25°C at pH 4.4 and that denaturation is fully reversible. NK-2H was found to have relatively little α -helical content. No dramatic change in the CD spectra was observed on addition of an oligodeoxynucleotide with a high-affinity NK-2 binding site. NK-2H uniformly enriched with ^{15}N was examined by 2D and 3D NMR. The results suggest that NK-2H has a novel homeodomain secondary structure.

Genes Expressed In The Developing Nervous System. Transposition of a P-element that contains the β -galactosidase gene from 1 site in the Drosophila genome to another yielded many transgenic fly lines that express β -galactosidase only in the nervous system during embryonic development. The developmental time and location of β -galactosidase expression then is determined by regulatory signals of the genes that contain the inserted P-element DNA. DNA flanking the P-element insertion sites were cloned from 15 of the most interesting transgenic fly lines and corresponding cDNA clones were obtained and were sequenced partially. Clone 393C-2 was shown to encode Drosophila high-mobility-group protein D (HMG-D), a DNA binding protein. A homologous mammalian protein, HMG-1, recognizes DNA conformation rather than nucleotide sequence; HMG-1 binds to cruciform DNA and to DNA with axial distortion due to cisplatin. The functions of HMG-1 and HMG-D proteins have not been identified; however, the proteins are thought to play a role in chromatin structure. Also the HMG domain has been found in many DNA binding proteins that regulate transcription. We find that the HMG-D gene is expressed ubiquitously during early embryonic development but later in development is expressed exclusively in the nervous system. The homozygous P-element insertion is a lethal mutation and is accompanied by striking morphologic defects in the central nervous system.

Clone 367C-3 DNA corresponds to a gene that encodes a novel zinc finger protein that is expressed in the CNS and anterior sensory organs. The homozygous P-element insertion is a lethal mutation that results in extraordinary morphologic defects in the ventral nerve cord of developing embryos. Clone 7D3C-1 corresponds to a novel Drosophila gene that encodes a member of the kinesin heavy chain gene family. Kinesin functions as a molecular motor for axonal fast transport of organelles or cell membranes on microtubule tracks from soma of neurons towards axon tips. Clone 314-4C-2 encodes a protein that is similar to the human QM protein, an apparent suppressor of Wilm's tumor, a pediatric nephroblastoma. Sequence analysis of cDNAs from other transgenic fly lines suggest that the cDNAs correspond to novel genes expressed in the nervous system.

Mammalian Homeobox and POU-Domain Genes. Approximately 8 kb of a novel mouse homeobox gene, NKx-1, a homolog of the Drosophila NK-1 homeobox gene, was sequenced. The amino acid sequences of the NKx-1 and NK-1 homeodomains differ by only 3 of the 60 amino acid residues. Both proteins also contain an acidic domain. However, most of the other regions of the protein that have been defined

differ markedly. NKx-1 poly A⁺ RNA was found to be most abundant in 10-day mouse embryos; the abundance progressively decreases thereafter. Northern analysis of poly A⁺ RNA from adults revealed 1 major band of NKx-1 poly A⁺ RNA in brain and trace bands in RNA from testes or spleen. The NK-1 gene is expressed in discrete regions of 14-day old mouse embryo mesencephalon and myelencephalon and also in spinal cord, vertebrae, and ribs.

A mouse genomic DNA library was screened with oligodeoxynucleotide probes for novel homeobox genes. Seventy-two positive recombinants were cloned. Thus far five novel homeobox genes have been found. Restriction site analysis of the 72 clones revealed additional classes of clones that have not yet been sequenced. In addition, novel POU-domain genes related to Brain-3 POU-domain cDNA were cloned from mouse and human genomic DNA and were sequenced. Two additional, novel human POU-domain genes related to Oct-3 were cloned and the POU-domain regions were sequenced. Sites of expression of Brain-1, Brain-2, Brain-4, and SKIP POU-domain genes in the mouse nervous system were determined by in situ hybridization as a function of mouse embryo developmental age and also were defined in the adult mouse. Hox 4.1 cDNA and genomic DNA were cloned and the complete Hox 4.1 open RNA reading frame was sequenced.

Regulation of a Calcium Channel α -1 Subunit Gene. The α -1 subunit of a voltage-sensitive calcium channel previously was shown to be inducible in NG108-15 cells and the expression of the gene was shown to control the ability of the cells to form synapses with striated muscle cells. The 5' -upstream regulatory region of the calcium channel gene was cloned and sequenced. A nucleotide sequence was found that is a powerful activator or an enhancerless chloramphenicol acetyltransferase reporter gene. A protein was found in NG108-15 nuclei that specifically binds to the activating sequence. A cDNA expression library in λ gt11 was screened for recombinants that direct the synthesis of proteins that bind to the nucleotide sequence and 35 positive clones were obtained. Seven kinds of clones were found that encode proteins that bind to oligonucleotides with appropriate sequence specificity but differ in specificity for double-stranded DNA, or (+) or (-) single-stranded DNA. Further work is needed to determine whether one or more of these proteins regulate the expression of the Ca²⁺ gene channel.

Enhancer and Promotor Selection. During the past year further work has been done on the selective amplification of DNA clones that contain enhancer or promoter nucleotide sequences that activate gene expression. The method is based on the observation that the synthesis of polyoma virus DNA in mouse cells requires viral enhancer sequences that also are required for the synthesis of mRNA from polyoma genes. Mouse genomic DNA fragments were ligated to polyoma DNA that lack the enhancer region of the virus. The E. coli origin of replication and β -lactamase gene also were inserted in the polyoma coat protein gene. Promoters or enhancers in the mouse genomic DNA inserts that activate plasmid DNA synthesis in mouse cells are able to replicate and hence are selectively amplified; whereas, plasmids that lack functional enhancer sequences do not replicate. Plasmid DNA was harvested from mouse cells that had been transfected and incubated for several days. Recovered DNA then was

amplified in *E. coli*. The selection method is highly effective; some clones were shown to increase in abundance more than 100,000-fold. Fragments of the recovered DNA inserts were shown to bind proteins from nuclei and to activate the expression of an enhancerless chloramphenicol acetyltransferase reporter gene. Previously, cDNA clones were obtained that encode proteins that specifically bind to oligonucleotide sequences that were identified by the oligonucleotide selection method. Partial sequences of some of the cDNA clones were obtained.

Differentiation of Excitable Membranes and Myofibrils of Striated Muscle. Ventral horn neurons from fetal rats or mice were cocultured with rat striated myotubes. Neurites induce accumulation of acetylcholine receptors on the myotube surface where neurites contact the muscle cells and also reduce the concentration of acetylcholine receptors where neurites contact a pre-existing patch with a high receptor density. Steps in the assembly of functional triads in cultured skeletal myotubes were studied by calcium imaging, immunocytochemistry, and electron microscopy. Developing triads and punctate structures containing ryanodine receptors (calcium release channels) and dihydropyridine receptors were found in 3-day old myotubes. Excitation-contraction coupling was found in cells with only a few of these structures. Changes in the maximal calcium release and uptake rates were observed during the course of myotube development that were correlated with the elaboration of the sarcoplasmic reticulum around the myofibrils.

Polyclonal antibodies were obtained directed against a protein from fetal pig brain that induces the formation of acetylcholine receptor aggregates on skeletal muscle cells in culture. The antibodies immunoprecipitate all acetylcholine receptor aggregating activity in crude fractions of brain and spinal cord extracts as well as in purified preparations and recognize a protein with a molecular weight of 120,000.

Genes Associated with Programmed Cell Death. During embryonic development, normal cell turnover, and other physiological and pathological processes, cells die by the activation of a mechanism of self-destruction, termed apoptosis or programmed cell death. The available information shows that apoptosis often requires gene activation and the synthesis of proteins presumably needed for cell death. Radiation and chemotherapeutic agents also elicit cell death by apoptosis. Since little is known about the genes or biochemical mechanisms involved in apoptosis, a study was initiated to identify genes involved in apoptosis in radiation-sensitive fetal rat brain neuroblasts following radiation treatment. It is anticipated that among the genes activated by irradiation will be genes required for neuroblast apoptosis, either for the triggering or for the execution of this process.

Fetal 17 day-old rats were irradiated (or sham-irradiated) with sublethal neutron/gamma irradiation in utero, and RNA isolated from brains 5 and 24 hours later was used to construct a subtractive cDNA library enriched in sequences of transcripts increased by irradiation. Clones from this library were screened and analyzed by

differential colony and Northern blot hybridization. At least 76 out of 682 analyzed clones were found to represent transcripts increased in abundance by irradiation, generally by a factor of 2-3 but much greater for at least one transcript. Sequencing of the inserts of these clones is in progress. Some clones were found to have previously unreported insert DNA sequences, while others represent ubiquitin, ferritin light chain, 12.3 protein (related to G- β proteins) and subunits of mitochondrial NADH dehydrogenase. These results indicate that a variety of transcripts are increased moderately during radiation-induced neuroblast cell death. Additional more strongly activated genes, which are expected in the triggering of cell death, are being sought currently.

Sequence and Characterization of the Hox A7 Gene. The Hox A7 gene is a mouse gene characterized by the presence of a homeobox region typical of the Antennapedia class of proteins found in Drosophila. Starting with a clone derived from mouse genomic DNA, the complete sequence of the structural gene, as well as upstream and downstream regulatory regions, were determined. A variety of studies have led to the assignment in this sequence of the probable transcription start site as well as likely regions for the interaction with proteins that regulate transcription.

Site-specific Mutagenesis of Presumptive ATP Binding Sites in Escherichia coli Adenylyl Cyclase. The region of the enzyme bounded by Alanine 190 and Arginine 197 was studied as a probable region for substrate binding. Site-directed mutagenesis was carried out on Lysine 196 by replacing this residue with a variety of other amino acids. It was found that replacement with other basic amino acids led to retention of some activity, while replacement with other classes of amino acids generally led to abolition of activity. The conclusion from these studies is that the charge in the vicinity of Lysine 196 is crucial for enzymatic activity.

Sequence and Organization of a Monocistronic ptsH Operon in Mycoplasma. A sequencing project, designed to isolate genomic clones for the enzymes of the transport system known as the phosphoenolpyruvate:sugar phosphotransferase system (PTS), was undertaken. Several clones were isolated that allowed the determination of the sequence of the gene encoding the PTS phosphocarrier protein known as HPr as well as of flanking regions upstream and that the gene encoding HPr (the ptsH gene) was located in an operon that is monocistronic. This is in marked contrast to the organization of all previously sequenced ptsH genes in other organisms where the ptsH gene is found in a polycistronic operon.

SUMMARY REPORT OF THE LABORATORY CHIEF

Laboratory of Biochemistry, NHLBI

October 1, 1992 to September 30, 1993

Section on Enzymes

The modification of proteins by reactive oxygen species is associated with a number of pathological conditions including aging, atherosclerosis, diabetes, arthritis, and pulmonary disorders. Studies in the Section on Enzymes are concerned with elucidation of the mechanisms of protein modification elicited by exposure to ozone, to the lipid peroxidation product, 4-hydroxy-2-nonenal, and to metal catalyzed mixed-function oxidation reactions.

Reactions of Proteins with 4-Hydroxy-2-nonenal. The cytotoxicity associated with lipid peroxidation is due in part to the formation of 4-hydroxy-2-nonenal (HNE) which was shown by other workers to react with cysteine residues and by studies in the Section on Enzymes to react also with histidine and lysine residues of proteins; this leads in the case of enzymes to inactivation. To facilitate further investigations on the role of lipid peroxidation in atherosclerosis and other pathological states, a highly sensitive immunochemical procedure was developed for the detection and quantitation of HNE-protein conjugate. Using this method, it was established that HNE-protein adducts are generated when crude rat liver homogenates are treated with HNE or are subjected to oxidative stresses such as exposure to tert-butylhydroperoxide or to metal catalyzed oxidation systems.

Ozone-Mediated Oxidation of Tryptophan Residues in Protein. Tryptophan residues are among the most sensitive targets for destruction by ozone. It was found that the destruction of tryptophan and of tryptophan residues in peptides by ozone is accompanied by spectral perturbations characterized by isobestic points at 228, 266, and 293 nm. This attests to the stoichiometric formation of a single product or to the production of multiple products in a constant molar ratio. From analysis of reaction mixtures, it was established that the degradation of tryptophan leads to formation of N-formylkynurenine, kynurenine, and an as yet unidentified product, characterized by an absorption band at 230-235 nm ($\epsilon = 7000$, $M^{-1}cm^{-1}$) in the molar ratio of 0.26, 0.04, and 0.7, respectively.

Age-Related Increase in Retinyl Palmitate. Lipofuscin is a general term for a material exhibiting a characteristic greenish-yellow fluorescence which accumulates in cells with age. A substance having similar properties was found to accumulate in rat liver as a function of age. This material was isolated as a pure compound and by means of proton NMR spectrometry and direct comparison with an authentic sample was positively identified as retinyl palmitate. The concentration in rat liver was found to increase almost linearly throughout the entire lifetime from 0.26 to 1.77 mg/g. As reported for lipofuscin, retinyl palmitate accumulated in the intracellular granules. It could, however, be separated from the particulate fraction by extraction with methanol.

Degradation of Active Oxygen Damaged Protein by the Multicatalytic Proteinase. The intracellular accumulation of oxidized or otherwise damaged protein reflects the balance between a multitude of factors that provoke damage, on the one hand, and degradation of the damaged protein by the multicatalytic proteinase (MCP), on the other. The MCP possesses a multiplicity of separate catalytic centers, the activities of which can be differentially inhibited by type-specific inhibitors. It was demonstrated that the trypsin-like, chymotrypsin-like and the peptidyl-glutamyl hydrolyzing activity of MCP each contribute to the degradation of damaged forms of glutamine synthetase and glucose-6-P dehydrogenase preparations obtained by exposure to metal catalyzed oxidation systems, whereas the chymotrypsin-like activity of MCP was not utilized in the degradation of these enzyme

following their inactivation by ozonolysis. Though preliminary, these results suggest that the MCP relies upon a battery of different proteolytic activities to recognize and degrade variously modified (damaged) forms of proteins.

Section on Metabolic Regulation

Protein Ubiquitination. Protein ubiquitination has been implicated in numerous intracellular processes, such as protein turnover, DNA transcription, etc. The enzymes involved including the ubiquitin activating enzyme, E1, the ubiquitin carrier enzyme, E2, which transfers the activated ubiquitin to its ligase, E3, or directly ubiquitinates its target proteins. Human E1 exists as two isoforms and can be phosphorylated *in vitro* by protein kinase C and *in vivo* by an unknown kinase. E2_{20kDa} and E2_{32kDa} are phosphorylated by a novel kinase which is being purified from HeLa cells and by a tyrosine kinase, respectively. Phosphorylation of E1 and E2_{32kDa} resulted in doubling of their activities. Together, the data show that protein ubiquitination appears to be regulated by reversible phosphorylation. To study the possible role of ubiquitin in DNA transcription, we found that transcription factors fos and jun can be multiple ubiquitinated with different pathways by the E2_{20 kDa}·E2_{32kDa} and E2_{14kDa}/E3 β system. However, only those ubiquitinated by E2_{14kDa}/E3 β system are degraded by the 26S protease.

Cytosolic Ca(II) Oscillation. In response to external stimulant, many cells release Ca(II) in an oscillating manner. To investigate whether reversible phosphorylation is a required process, we found that histamine induced Ca(II) oscillation in HeLa cells exhibits (i) no effect by cAMP-dependent protein kinase; (ii) while PMA, a protein kinase C activator, abolished the oscillation, a PKC inhibitor staurosporine was able to overcome the PMA effect, suggesting that PKC exerts its effect by phosphorylation of a factor other than the IP₃ receptor or ER Ca-ATPase; (iii) calmodulin-dependent protein kinase II plays a major role and it is required for Ca(II) oscillation by regulating the ER Ca(II)-ATPase. However, phosphorylation of the IP₃ receptor by the kinase may also occur.

Characterization of Mg(II)-Dependent, Ca(II)-Inhibited Phosphatases. A new Mg²⁺-dependent phosphoserine/threonine protein phosphatase from bovine brain has been identified as a 78,000 molecular weight monomeric protein. Substrate specificity and inhibitor susceptibility establish that the enzyme is different from any other known protein phosphatase. Another protein of the same molecular size copurifies with the phosphatase and, when phosphorylated by protein kinase C or casein kinase II, serves as a good substrate for the phosphatase. Amino acid sequence and composition analyses indicate that this substrate is a homolog of a human leukemogenic protein, SET. The phosphatase may play a major role in cell proliferation.

On the Protective Mechanism of the Thio-Specific Antioxidant Enzyme (TSA). A TSA, purified and cloned by Rhee and coworkers, is known to protect against oxidative modification by thiol-induced oxidants. Using EPR and the spin-trap method, we showed that TSA exerts its protection by scavenging thiyl radicals before they generate more reactive radicals. However, present data cannot rule out the possibility that TSA can also use a broad range of substrates.

Thiyl Radical Formation in Cells. Using NCB-20 cells, we demonstrated the formation of glutathionyl radicals when these cells were subjected to oxidative stress by exposing them to H₂O₂ produced by glucose/glucose oxidase. This is the first illustration that glutathione functions as a first line of defense under oxidative stress by scavenging free radicals and form glutathionyl radicals which can be removed by TSA.

Reaction of α -Ketoaldehyde and Proteins. α -Ketoaldehyde is a product of amino acid oxidation. This compound may be involved in glycation of proteins and contributes to aging processes. We showed that methylglyoxal can cross react with Cu,Zn-superoxide dismutase and cause ~70% decrease in its enzymic activity.

Effect of Electric Fields on Biomembranes. When intact cell membrane is subjected to some critical electric field strengths, it becomes transiently permeabilized. The susceptibility of membranes to this dielectric breakdown is found to be dependent on the field strength, direction of the electric field, and the resting membrane potential.

Section on Protein Function in Disease

Research in this section focusses on metal-catalyzed oxidative modification of proteins, a covalent modification which has been implicated in important physiologic and pathologic processes. These include the aging processes, arthritis, atherosclerosis, gene expression, hypertension, intracellular protein turnover, oxygen toxicity, and reperfusion injury after ischemia. Research goals include determination of the chemical and structural changes induced by oxidation; identification of the processes affected by oxidative modification; purification and characterization of the systems which catalyze the modification and subsequent proteolysis; understanding the controls which may regulate the modification and proteolysis of specific proteins; and application of this knowledge to the rational design of irreversible enzyme inhibitors.

In the last year, emphasis was placed in three areas: (1) the role of oxidative modification in causing tissue damage during oxidative stress; (2) detection and quantitation of oxidatively modified proteins; and (3) inhibition of the protease from the human immunodeficiency virus.

Endothelial cells line all blood vessels, and alterations in the endothelium have been linked to acute disorders such as ischemia-reperfusion injury and to chronic diseases such as atherosclerosis and hypertension. Utilizing cultured bovine endothelial cells, we examined the time course of alterations which occur upon exposure to hydrogen peroxide as an oxidative stress. Cell viability drops as a result of the exposure, but only after several hours. The energy charge of the cell drops distinctly earlier, with ATP concentrations decreasing within 15-30 minutes after exposure. However, oxidative modification of cellular proteins occurs within 1 or 2 minutes, essentially as fast as measurements allow. Enzyme activity of key metabolic enzymes drops almost as quickly, consistent with their oxidative inactivation. Thus, one of the earliest events during oxidative stress is modification of cellular proteins. The drop in cellular energy occurs later, presumably as a consequence of oxidation of structural and catalytic proteins.

Studies of oxidative modification *in vivo* require sensitive assays which can detect and characterize oxidative modifications in samples containing small amounts of protein. Two methods were successfully established. The first is a sensitive assay for both HPLC and immunochemical detection of oxidatively modified proteins. The immunochemical method allows detection of picomole quantities of oxidized proteins, making it sensitive enough for application to human fluids or biopsy specimens. The second method facilitates identification of the specific residues which are oxidized in sensitive proteins. It is based on sequence analysis of the complete peptide collection of control and oxidized proteins, with cleavage and sequencing performed *in situ* on a resin. Comparison of the cycle yields allows identification of the altered residues.

As an outgrowth of studies, it was found that low concentrations of copper were able to inactivate the protease from the human immunodeficiency virus. Investigations this year

have established that a copper containing peptide is also capable of inhibiting the protease. The copper-peptide is the well-characterized liver growth factor, glycine-histidine-lysine. This finding supports the feasibility of developing a new class of protease inhibitors which are not directed to the substrate-binding site.

Section on Intermediary Metabolism and Bioenergetics

In humans and animals, the trace element selenium (Se) is a key component of certain essential enzymes that control development and protect tissues from reactive organic peroxides. Recognition that these enzymes contain Se in the form of selenocysteine was based on earlier chemical studies in this laboratory on the selenoprotein A component of clostridial glycine reductase. Recent studies in prokaryotes on the mechanism of biosynthesis of selenocysteine and its insertion into proteins have shown that the reactive Se donor compound required for the process contains Se bonded to phosphorus and is indistinguishable from chemically synthesized monoselenophosphate. This highly oxygen-labile compound also is required as Se donor for conversion of 2-thiouridine residues in tRNAs to 2-selenouridine. The presence of Se in the anticodons of lysine, glutamate, and glutamine tRNAs affects codon-anticodon interaction and thus has a regulatory role. The bacterial selenoenzyme nicotinic acid hydroxylase lacks selenocysteine and instead contains Se in a dissociable cofactor. EPR studies show that the Se is coordinated to a Mo(V) radical species present in the molybdopterin cofactor of the enzyme. This was verified using enzymes containing the stable isotope ^{77}Se or ^{95}Mo in place of normal isotope abundance species. Moreover, enzyme containing S instead of Se showed a very low EPR signal characteristic of Mo(V)-Se coordination. A Se and molybdopterin-dependent formate dehydrogenase from *E. coli* exhibited the same Mo(V)-Se signal, whereas a mutant S form of the enzyme having low catalytic activity showed almost no signal. In the latter mutant protein, cysteine replaced the essential selenocysteine residue. Thus, in these enzymes, protein-based radical species involving Se and Mo are implicated as participants in the reaction mechanisms.

Studies on the mechanism of selenophosphate synthesis from ATP and NaSeH using mutant enzyme forms produced by site-specific mutagenesis revealed that cysteine-17 and lysine-20 are essential for selenophosphate synthetase activity, but not for binding of ATP. The reaction products--SeP, Pi, and AMP--suggest that Enz-PP might be an intermediate in the catalytic process.

In the glycine reductase reaction the two carbons of glycine are transferred to selenoprotein A forming a CM-Se derivative of the selenocysteine residue. Reductive cleavage of this selenoether converts the CM group to an acetylthiol ester derivative of protein C. Marked specificity of this reaction that requires protein-protein recognition is evident from the finding that selenoprotein A and protein C from different clostridia are not interchangeable. Earlier designation of selenoprotein A as a glycoprotein based on a positive test for carbohydrate after periodate treatment provided an explanation for specificity of interaction with protein C. However, the presence of glycosyl groups is now in doubt based on recent detailed studies. Instead, oxidative cleavage of the protein by periodate to give reactive carbonyl groups may be responsible for the positive carbohydrate test. Thus, other properties of proteins A and C determine specificity of interaction and mechanism of this novel energy conservation process that leads to generation of acetylphosphate and finally to ATP.

Section on Protein Chemistry

The Section on Protein Chemistry is studying the physical and chemical properties of macromolecules of biological interest and the roles of ligand binding and of protein-protein and inter- and intra-subunit interactions in enzyme catalysis and regulation. The energetics of ligand binding to proteins involve contributions from both ligand-protein and protein-protein interactions. Ligand-promoted changes in protein-protein interactions underlie the phenomenon of cooperativity in ligand binding to proteins and, in addition, give rise to many examples of stabilization and destabilization of protein structures by ligands and metal ions.

A goal of studies on protein folding is to understand the stabilization of complex secondary and tertiary structures in native proteins that leads to their unique conformations. Assembly processes and biological interactions depend on the correct folding of polypeptide chains. If an unfolding reaction can be described as a reversible, two-state transition, the thermodynamics of protein folding/unfolding can be determined experimentally from measuring the free energy of unfolding as a function of temperature. Differential scanning calorimetry (DSC) directly gives thermodynamic parameters and, in addition, provides some insight into the mechanism of unfolding a biopolymer. Isothermal calorimetry is used to measure stoichiometry and thermodynamic parameters of protein-ligand and protein-protein interactions. Spectral techniques are used to monitor changes in secondary structure and the environment of tryptophanyl and tyrosyl residues (which may be in the same or different protein domains). Ultracentrifugation, HPLC gel-filtration, and light scattering give information on changes in the size and shape of biopolymers.

Last year, we reported on the thermally induced reversible, partial unfolding transitions of dodecameric Mn•glutamine synthetase (622,000 M_r) from *E. coli* at pH 7. We showed that each subunit has two domains of slightly differing stability ($\sim 2^\circ\text{C}$ with a midpoint at 51.6°C) and that cooperative interactions link partial unfolding of all subunits within the dodecamer. However, the overall enthalpy change for this reversible thermal transition is only a small fraction (1-4%) of that estimated for complete unfolding. Currently, we are studying urea and guanidine•HCl-induced denaturation and at 2 M urea, we can observe both the reversible transition ($t_m \sim 45^\circ\text{C}$) and much larger overlapping endotherms at ~ 58 , 78 , and 91°C . Thermally induced unfolding of *S. typhimurium* tryptophan synthase (143,000 M_r) and its isolated subunits also is being studied at pH 8 in order to elucidate the overall conformational stability of this $\alpha_2\beta_2$ multienzyme complex and to characterize intramolecular subunit interactions. DSC and spectral data are consistent with a model of six sequential two-state transitions for the $\alpha\beta\beta\alpha$ complex with bound pyridoxal 5'-phosphate: at $<65^\circ\text{C}$, there is a partial exposure of Trp177 in β chains followed by sequential unfolding of α chains and at $>70^\circ\text{C}$, β chains undergo unfolding transitions. The cofactor increases both the linkage between unfolding domains and the stability of $\beta\beta$ subunits. In addition, phospho- and dephospho-myosin-II from *Acanthamoeba* have been found to have identical DSC profiles: a single sharp endotherm at $\sim 42^\circ\text{C}$. An ATP analogue uncouples the unfolding of the rod and head portions of myosin-II and work is in progress to identify thermodynamic domains. In other studies, a thermodynamic characterization of the influenza virus hemagglutinin (which is a trimer of 220,000 M_r) has revealed proton-induced conformational changes that may relate to physiological function. Isothermal titration calorimetry of the catalytic trimer (c_3) of aspartate transcarbamoylase from *E. coli* with a Zn-domain, C-terminal regulatory chain (r) fragment (53% of native r M_r) showed that there is strong energetic coupling between substrate binding and the stability of r:c contacts, which maintain allosteric properties of the intact enzyme.

Section on Signal Transduction

The binding of hormones, peptide growth factors, neurotransmitters, and immunoglobulins to their cell surface receptors results in the activation of inositol phospholipid-specific phospholipase C (PLC), which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P₂) to two second messenger molecules, diacylglycerol and inositol 1,4,5-trisphosphate. Studies from our and other's laboratories suggest that at least two distinct types of transducer molecules, protein tyrosine kinases (PTKs) and G proteins, participate in the coupling of receptor binding to PLC activation. Complexity of the transducing mechanisms is increased by the existence of multiple isoforms of PLC. Six mammalian isozymes, which can be divided into three types (β , γ , and δ) on the basis of primary structure, have previously been identified at both protein and DNA levels.

During the period covered, we uncovered two more β -type isozymes and named them PLC- β 3 and PLC- β 4. We studied the activation of PLC- β 1, PLC- β 2, and PLC- β 3 by α subunits of G_q class G proteins. The extent of activation decreased in the order of PLC- β 1 \geq PLC- β 3 \gg PLC- β 2, suggesting a certain degree of specificity in the interaction of G_q α subunits with different PLC- β isozymes. The region required for activation by G_q α was localized to the 45-kDa carboxyl-terminal region of PLC- β 1.

None of the G_q protein α subunits is a substrate for ADP-ribosylation by pertussis toxin. It has been suggested that $\beta\gamma$ subunits, rather than α subunits, might be the active entity of pertussis toxin-sensitive G-proteins that have been shown to be involved in receptor-mediated activation of PLC. We demonstrated PLC- β isozymes as the target of $\beta\gamma$ subunits: Bovine brain $\beta\gamma$ subunits activated PLC- β isozymes in the order of PLC- β 3 $>$ PLC- β 2 $>$ PLC- β 1, which differs from the order of PLC- β 1 \geq PLC- β 3 \gg PLC- β 2 for G_q α -dependent activation.

Furthermore, the half-maximal concentration of $\beta\gamma$ (25 nM) required to activate PLC- β 3 is much higher than that of G_q α subunits (0.6 nM) required to activate PLC- β 1. These results suggest that the extracellular signals that induce the dissociation of G_o or G_i, the pertussis toxin-sensitive G proteins abundant in brain, should enhance the hydrolysis of PtdIns 4,5-P₂ in brain primarily through activation of PLC- β 3 (PLC- β 2 is not detectable in brain). However, signals that activate the less abundant G_q class heterotrimers should result in the activation primarily of PLC- β 1 and PLC- β 3 by the corresponding α subunits.

The γ -type isozymes of PLC, PLC- γ 1 and PLC- γ 2, are activated as a result of phosphorylation by PTKs. Phosphorylation has been shown to be catalyzed by either growth factor receptors that possess ligand-activated cytoplasmic PTK domains or by unidentified nonreceptor PTKs linked to a variety of cell surface receptors. Recently, we demonstrated that activation of Fc γ RIII, the receptor involved in the antibody-dependent cellular cytotoxicity in natural killer cells, is coupled to a nonreceptor PTK, which phosphorylates and thereby activates PLC- γ 1 and PLC- γ 2. The phosphorylation of PLC- γ 1 and PLC- γ 2 by src-family PTKs lck, lyn, hck, fyn, and src was studied *in vitro*. All five PTKs phosphorylated PLC- γ 1 and PLC- γ 2 without any distinct specificity between PLC- γ 1 and PLC- γ 2, or between the five PTKs.

ANNUAL REPORT OF THE
October 1, 1992-September 30, 1993
LABORATORY OF BIOPHYSICAL CHEMISTRY
CHEMICAL STRUCTURE SECTION
STRUCTURAL BIOPHYSICS SECTION
STRUCTURAL NUCLEAR MAGNETIC RESONANCE
BIOPHYSICAL INSTRUMENTATION

As mentioned last year, the Chemical Structure Section specializing in mass spectrometry, has switched its emphasis to proteins, peptides and oligosaccharides. The newly acquired electrospray triple quadrupole mass spectrometer as well as the 600 Mhz nuclear magnetic resonance spectrometer have been fully operational for nearly one year and have produced much interesting data. A new matrix assisted laser desorption ionization mass spectrometer is due to arrive shortly.

The areas currently under study and development by the six senior members of the Laboratory include peptide, protein, and oligosaccharide mass spectrometry (Fales), small molecule high resolution x-ray crystallography, scanning tunnelling and force field microscopy (Silverton), nuclear magnetic resonance, computer modeling and in vitro protein synthesis (Ferretti), biocalorimetry and near infrared spectroscopy (Berger), and countercurrent chromatography (Ito).

Ferretti continues his studies on peptides and small proteins in the free and bound states in an effort to understand conformation-activity relationships. Solution and solid state nuclear magnetic resonance (NMR) spectroscopy is being used along with ancillary techniques of circular dichroism spectropolarimetry, microelectrophoretic mobility, fluorescence spectroscopy, differential scanning calorimetry, mass spectrometry, low angle neutron scattering and computer modelling.

Recently he has completed about 75% of the 3-D structure of the 77 amino acid homeodomain encoded from the homeobox gene of *Drosophila melanogaster*. Based on sequence homologies it has been hypothesized that the structure of the homeodomain of eukaryotic gene regulatory proteins includes a helix-turn-helix motif designed for sequence-specific DNA-binding. However, he has found that the secondary structure appears to utilize a helix-turn- β -pleated sheath to bind to the DNA. This represents an entirely new binding motif for homeodomain proteins. Furthermore, the protein binds to DNA sequences that contain AAGTGG as the core sequence with a dissociation constant of 10^{-10} . This result contrasts with those homeodomain proteins that contain the helix-turn-helix motif which bind to DNA sequences that contain TAAT as the core sequence. His NMR studies on the protein-DNA complex show that the binding involves one NK-2 molecule for each DNA molecule.

In continuing work on the binding epitope of the viral coat protein of Rift Valley Fever Virus, several single sight ^{15}N enriched peptides have been synthesized to study the peptide in the antibody bound state. Studies have been carried out on the minimal binding epitope, an octapeptide whose sequence is Tyr-Lys-Gly-Thr-Met-Asp-Ser-Gly and Phe-Glu-His-Lys-Gly-Gln-Tyr-Lys-Gly-Ser-Met-Asp-Ser-Gly-Gln-Thr-Lys-Arg-Glu-Leu-Lys. The interpretation of the NMR spectra is tentative at this point with some spectra suggesting that the peptide might be binding the antibody in two distinct conformations.

His studies also continue on the 828-848 fragment from the intraviral domain of the HBX2 isolate of gp41, the outermost end of the HIV membrane protein. He is attempting to determine the three-dimensional structure of the peptide in micelles and bound to a model membrane surface by carrying out solid state Deuterium NMR studies.

All of the above studies require rather large amounts of the peptides and proteins involved and this year his group has developed a computer process control for their continuous flow in-vitro translation system discussed last year. As a test protein, globin was translated successfully and now other proteins, such as the class I major histocompatibility complex with its bound peptide, are being synthesized.

In other NMR work Highet continues his studies of the oxidative degradation of cytochrome P450 and related materials. Oxidation of myoglobin by 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine provided a product in which the delta-meso proton has been replaced. However, the results from homonuclear correlation spectra suggest structures which are not compatible with the chemical shifts. It is hoped that studies by heteronuclear multiquantum methods will elucidate this discrepancy.

In a collaboration with Prof. G. J. Kapadia of Howard University, he is elucidating the structures of a series of hydroxyanthracenes of natural and synthetic origin which evince an antitumor promoting effect on Epstein-Barr virus.

He has also elucidated the structures of a series of food dyes from the FDA that had been separated by countercurrent chromatography.

Finally, he is preparing a definitive review of the chemistry of the antimalarial sesquiterpene artemisinin. Because the action of this material depends on a novel endoperoxide function, artemisinin promises to be a powerful means to combat malarial resistant to established drugs.

Silverton has completed the determination of the absolute configuration and conformation by x-ray crystallography of many compounds of interest at NIH These include: the HIV drug

adenallene; R-quinucleodiny-R-iodobenzilate, a drug used in *in-vivo* imaging by photoemission tomography; the enzyme inhibitor 10- β -aziridiny testosterone; polyzonamine, an intermediate in the synthesis of a toxin from dendrobatid frogs; a CNS-active compound from Indian folk medicine and an unusual metal complex. In a newer activity, Silverton is examining the possibility of using force field microscopy to determine the structure of the envelope of a protein from a crystal. If possible, the x-ray method could then be applied directly without making derivatives of the protein. Early studies have examined the problem using ammonium hydrogen tartrate as a model, but the reality of the images obtained is still in question.

Ito, in pursuing the subject of pH-zone countercurrent chromatography, has successfully separated several FDA food dyes into their components. This new technique operates at the multigram level and supplies compounds of exceedingly high purity as a series of rectangular peaks that are cleanly separated. The technique is related to frontal, or displacement, chromatography, an old technique that is undergoing a renaissance at present because of its simplicity and ability to concentrate trace quantities. Using DNP-amino acids as models, Ito has now completed the theory of this interesting technique.

Ito has also applied his cross axis centrifuge to the separation of a series of proteins and found that, while they do separate, their efficiencies (peak shape) are low. He ascribes this either to their size or heterogeneity. In a practical example, he has separated several peptides from an E. coli lysate, obtaining fractions that show single spots on gels.

With an improved version of his coil-planet centrifuge, Ito has successfully separated rat and mouse peritoneal cells. Thus rat mast cells were separated at a purity of 99.1% in 42% yield and 90% pure mouse mast cells in spite of their low population in the sample. All cells were found to be biologically intact.

Berger, using his microcalorimeter, has determined the dependence of CO₂ binding to hemoglobin as a function of chloride concentration. A drop-off in the enthalpy of the CO₂-deoxy Hb interaction begins at 0.01M chloride and continues downward at high concentrations of chloride (1.00M). This result is consistent with the fact that CO₂ and chloride are competitive allosteric effectors binding to the same site(s) or nearby site(s). ¹³C NMR measurements gave binding results which agreed within experimental error with previous work.

In other work, Berger has developed a method for the simultaneous analysis of ATP, ADP, and P_i in the 1 to 25 micromolar regime with <5% error using linear regression with six variables. The aim of this work is to fully characterize all of the intermediates in the key biological reaction, the hydrolysis of ATP.

Fales continues to study the ion trap as a unique mass spectrometer. In understanding its spectra he has found it necessary to consider the phenomenon of self-CI which strongly affects the spectra at higher sample loads. The instrument has recently been adapted to achieve much higher mass and resolution suitable for protein and peptide analysis, however the conversion is not as straightforward as advertised.

Use of the triple quadrupole has expanded considerably and several problems have been solved. In one case, prothymosin, a protein essential for cell division, was found to be phosphorylated in a single position at the N-terminal acetylserine by a comparison of the mass spectra of several peptides obtained by treatment with enzymes Lys-C and Asp-N. In another, a highly phosphorylated protein obtained from rat neural tissue, his group was able to identify the sites of phosphorylation among fifty potential positions. In still another case, they were able to determine the structure of a specific immunologically active glycan structure on a human secretory component. In several other cases the mass spectrometer has solved relatively simple problems merely by confirming molecular weights.

Using both NMR and Cf-252 mass spectrometry he has identified the site of phosphorylation on an unusual maltose derivative obtained from oral bacteria. The normal site is 1 but in this case it is 6' phosphorylated and a synthesis is currently underway.

An unusual finding by this group concerns the apparent activity of various RNAs in catalyzing the polymerization of long and short chain lactones into polyesters. This reaction is used by *Colletes thoracicus* in constructing its nest linings as reported several years ago when it was discovered that a substance in its salivary fluid was responsible for this action, but that the substance was not a proteinaceous enzyme.

Annual Report of the Laboratory of Cardiac Energetics

National Heart, Lung and Blood Institute
October 1, 1992 through September 30, 1993

The major goal of the Laboratory of Cardiac Energetics is to improve our understanding of the cellular and molecular processes involved in the conversion of energy to useful forms of work in the heart and other tissues. With this insight we hope to develop new strategies for the diagnosis, prevention and treatment of heart disease. Our technological approach to these problems is the use of non-invasive nuclear magnetic resonance (NMR) and optical spectroscopy techniques. These methods permit the non-invasive monitoring of several critical parameters of energy metabolism including metabolites, blood flow and tissue oxygenation in intact tissues or humans. The application of these technologies to humans allows us to evaluate these tools as non-invasive diagnostic modalities.

The major energy metabolism pathway we have been concentrating on over the last year is the complex interaction between energy conversion processes in the heart (i.e. oxidative phosphorylation), muscle contraction (i.e. pumping of blood) and coronary blood flow. For the heart to function properly, these three elements must be orchestrated with remarkable accuracy to provide the proper amounts of oxygen, substrates and energy in the appropriate form to support the pumping of the blood. Myocardial muscle contraction is believed to occur by the muscle utilizing the energy in adenosine triphosphate (ATP) produced predominately by oxidative phosphorylation occurring in the mitochondria and converting oxygen to water.

To use ATP for muscle contraction, ATP is hydrolysed to adenosine diphosphate (ADP) and inorganic phosphate (Pi). It was long believed that these hydrolysis products of ATP serve as the intracellular signals which regulate the rate of oxidative phosphorylation in the heart, while the further breakdown of ADP to adenosine was believed to regulate the resistance of the coronary blood vessels and thereby control the coronary blood flow. However, as we have previously demonstrated, the hydrolysis products of ATP, ADP and Pi do not change during physiological increases in work. That is, under conditions in which the turnover of ATP has increased almost 5-fold, the hydrolysis products of this reaction do not increase.

This indicates that the ATP, ADP and Pi levels are highly buffered by oxidative phosphorylation in the healthy myocardium and that these metabolites are unlikely to play an important role in the orchestration of metabolism or coronary blood flow. Hence some other cytosolic parameters must be responsible for the orchestration of these critical processes in the heart. Over the last year we have evaluated new sites where this regulatory processes could be occurring in the control of energy metabolism during work stress.

Previous studies on the control of oxidative phosphorylation in the intact heart suggested that the utilization of substrates may be a rate-limiting step. Indeed, we had proposed that the level of mitochondrial NADH, generated by substrate oxidation, may be a key controlling step in the regulation of oxidative phosphorylation.

To evaluate this hypothesis, we investigated the effects NADH on the kinetics of oxidative phosphorylation in isolated mitochondria. These studies revealed that the level of mitochondrial NADH is a strong regulator of the maximum velocity (V_{max}) of oxidative phosphorylation. The relationship between NADH concentration and V_{max} is essentially linear with a slope of approximately 2. NADH increases the V_{max} of oxidative phosphorylation with no effect on the apparent affinity of the reaction for adenosine diphosphate (ADP) measured with several reducing equivalent sources. These studies suggested that one or more elements of complex oxidative phosphorylation process are being up-regulated with an increase in NADH.

F1-ATPase is the enzyme which phosphorylates ADP using the electrochemical gradient across the inner mitochondrial membrane. Recent studies from Harris et al. have suggested that this enzyme may be appropriately regulated to participate in the control of oxidative phosphorylation in cultured myocytes. We therefore investigated whether increases in F1-ATPase activity are associated with the NADH-dependent increases in V_{max} observed in isolated mitochondria. A linear relation between the NADH stimulated V_{max} and the F1-ATPase hydrolysis activity was found, suggesting that the F1-ATPase is being modulated by NADH levels through some as-yet-undetermined mechanism. Our current hypothesis involves the inner mitochondrial membrane as the intermediary between NADH and F1-ATPase activity.

Following up on these results, we developed a new F1-ATPase assay that permits repeated biopsy assays from the dog heart in vivo. These studies revealed that the F1-ATPase activity is downregulated during hypoxia and ischemia as previously reported from isolated mitochondria studies. We also discovered that the activity is increased with alpha-agonist stimulation of cardiac work in vivo. These results are consistent with the notion that the regulation of F1-ATPase activity may play a key role in the short-term regulation of oxidative phosphorylation in the in vivo heart.

Though both the isolated mitochondria and in vivo heart studies on F1-ATPase indicate a significant correlation between the rate of oxidative phosphorylation and F1-ATPase activity, there are several problems with the interpretation of the data. The major problem is that in the in vitro assay of F1-ATPase activity the reverse, or ATP hydrolysis, reaction is being monitored. This reaction is the opposite of the one in which we are interested. Whether information can be construed from the hydrolysis reaction about the forward F1-ATPase reaction is a very controversial issue. Indeed, it is clear that an assay for determining the forward activity of the enzyme needs to be developed. The determination of the pertinent kinetic parameters is also required. Despite this controversy, the fact

that sustained modifications of the F₁-ATPase activity are being recorded after a variety of metabolic challenges suggest that this enzyme may be an important regulatory site which merits further study.

Our studies on the interaction of mitochondrial NADH and respiratory rate have continued in the intact heart. Dr. Heineman and Dr. Laughlin have demonstrated the same linear relationship between mitochondrial NADH and phosphorylation potential that was shown in isolated mitochondria by varying the metabolic substrate provided to the perfused heart. In extensive extraction studies these investigators have also shown that the cytosolic NADH redox state does influence the phosphorylation potential, but to a much smaller extent than the mitochondrial redox state.

Dr. Laughlin has been evaluating the effects of different substrates and hormones on the rate of glycogen synthesis, measured using ¹³C NMR in the canine heart with the goal of a better understanding of the mechanisms involved in substrate utilization in the intact heart. A surprising result in her studies was the discovery that infusions of lactate, pyruvate or ketone bodies to the local cardiac vasculature or systemically resulted in a large stimulation of glycogen synthesis. The degree of stimulation was larger than that attained with insulin infusions under identical conditions. Thus, cardiac glycogen synthesis is extremely sensitive to circulating substrate concentrations. The reason for this may involve the competition for glucose by oxidative pathways and glycogen synthesis.

One of the difficulties in evaluating interaction sites between two complex reaction systems such as muscle contraction, blood flow and metabolism is the complexity of these systems themselves. Where is a logical place to start? One attractive idea is to find a system where a transformation from one control network to another exists and look for the areas of the network which are modified. If such a transformation could be found, it may also be a power system to use the new molecular biology tools to determine the specific enzymes and proteins involved in the transformation. Such a system may exist in the chronic pacing transformation of skeletal muscle.

In this model, skeletal muscle is chronically stimulated in vivo for several weeks. After this period a transformation occurs in which the muscle takes on many of the characteristics of cardiac muscle. Most notable is the lack of alterations in ATP, ADP, P_i or creatine phosphate with large increases in workload, in stark contrast to control skeletal muscle. We have begun to evaluate this system to take advantage of this transformation to gain information on the potential metabolic control sites in the normal heart. Dr. Ryschon has developed an in vivo rabbit TA/EDL muscle preparation for this purpose. We have demonstrated that a appropriate transformation of this muscle occurs with chronic pacing and that a metabolic pattern exists similar to the heart with regard to a work stress challenge.

In the course of these studies, we also discovered that the muscle fatigue associated in normal skeletal muscle in vivo is not caused by excitation contraction failure as previously proposed, but due to a change in the metabolic/ mechanical efficiency of the muscle. Studies are now underway to evaluate the effects of work stress on intracellular Ca, NADH and oxygen levels as well as the activity of several key metabolic enzymes in the mitochondria. A more complete scan of the alterations in metabolic enzymes is proposed using mRNA detection techniques in the near future. Hopefully, this preparation will not only teach us about the transformation of skeletal muscle by training, but also provide important insights into the regulation of oxidative phosphorylation in the heart.

A large portion of our efforts over the last year have been devoted to the development of NMR techniques to the study of organ physiology and biochemical structure in vivo. These approaches are being developed to provide new tools in studying the function of the body in vivo, as well as potentially provide new non-invasive diagnostic tools to clinically evaluate humans.

Using a saturation transfer approach, in combination with standard magnetic resonance imaging (MRI) techniques, we have successfully imaged the rate of magnetization transfer between various macromolecule protons and protons in water in intact tissues. The contrast generated by this process, termed magnetization transfer contrast (MTC), is unique in magnetic resonance imaging and is currently being developed for clinical applications around the world. These applications include the study of multiple sclerosis, blood vessel angiography, AIDS, cancer and sports medicine. This approach has also provided a unique insight into the basic mechanisms of water proton relaxation in biological tissues which we have been concentrating on over the last year.

In our attempts to evaluate the molecular mechanisms responsible for the MTC effect, we have studied a large series of macromolecules and lipids. In summary, these studies have demonstrated that a surface hydroxyl group is the most effective surface group in generating this effect, while amine groups can provide a weak effect.

Our working hypothesis for the magnetic interaction of water with macromolecules involves interaction of water with a surface hydroxyl group. We are currently testing this model by using site-specific isotope replacements in macromolecules. These studies block the dipolar interaction at specific sites with deuterium to pinpoint the specific sites involved in this process. Using cholesterol, DPPG and DPPC, we have found that specific motional domains in the lipids can be probed using this approach. We have found that the protons surrounding the hydroxyl of the cholesterol molecule are not the only dipolar coupling sites and that significant transfer to the backbone of the adjacent lipid also occurs. Most surprisingly, long-range coupling could be detected to the acyl-chains of the lipid, suggesting the presence of short-lived water protons in this hydrophobic region of the bilayer.

These physiochemical studies are quickly improving our ability to interpret magnetization transfer phenomena observed in MRI scans of pathology.

Since November 1991 the Laboratory has been outfitting a 4.0 Tesla 1-meter bore NMR system for human studies. This is the highest magnetic field strength available for whole-body NMR studies. Only two other similar systems exist in the world. This high field has demonstrated that it will improve signal-to-noise ratios in spectroscopic studies of tissue biochemistry as much as 3-fold, as well as improve the spatial resolution (3-fold) and time resolution (7-fold) of NMR imaging studies. These advantages have made many new studies possible on humans which could not be performed at the more commonly available fields of 1.5 or 2.0 Tesla.

The magnet itself was constructed by Oxford Magnetics, while much of the other hardware was provided by General Electric. We have successfully added a research console to this system which permits us to program state-of-the-art NMR acquisition schemes in this prototype system. In addition, the entire front end of the system has been rebuilt and is currently operating at state-of-the-art in terms of signal-to-noise and radiofrequency performance.

In the last year there have been several major technical accomplishments. Mr. Han Wen, a physics graduate student from the University of Maryland, has invented a new NMR coil with excellent performance characteristics at 170 Mhz. This new design relies on the mutual inductance of many small circuits to overcome many of the problems associated with using high magnetic fields on large structures (>20cm diameter) on human subjects. This coil arrangement has permitted many of the first whole-head examinations at 4.0 Tesla. Currently, modifications of this system are underway to permit body and heart studies.

We have also designed and constructed a new arrangement to study the human heart using two opposing coils. This has permitted excellent radiofrequency penetration characteristics which surpass previous designs. However, the detection of EKG and associated large motion artifacts in the body at 4.0 Tesla have limited the applications to date. We are currently developing a retrospective gating scheme to overcome this major limitation along with sophisticated EKG filtering systems.

A major project in the Laboratory is the use of MRI techniques in the evaluation of tissue oxygen levels and blood flow. These studies deal with the development of methods for non-invasive monitoring of perfusion and blood oxygenation, and real-time imaging. The basic experimental tool is Echo-Planar Imaging (EPI), a type of magnetic resonance imaging which acquires images in 0.1 sec. or less, much faster than conventional MRI. The images can be sensitized to spatial variations in blood flow and blood oxygenation, and can be obtained at a rate of up to 1 per second. Other imaging techniques such as gradient-recalled echo and spin-echo have also been used for these purposes in heart and other tissues.

These studies can be broken down into two areas: cardiac and brain functional imaging. In the cardiac studies we are utilizing a canine heart model to evaluate the use of gradient-recalled echo techniques to monitor coronary blood flow and tissue oxygenation. Coronary blood flow alters the apparent spin lattice relaxation time (T_1) of the tissue water while changes in hemoglobin oxygenation alters the apparent spin-spin relaxation time (T_2). By using appropriate image acquisition schemes the relative contribution of blood flow and tissue oxygenation with millimeter resolution has been obtained in this animal model. We are currently beginning a series of quantitative validation studies and developing hardware requirements to conduct these studies on humans.

The ability to map brain metabolic responses also relies on these NMR relaxation consequences of blood flow and tissue oxygenation. Using this approach the location and time course of metabolic alterations in the brain in response to stimuli can be assessed with millimeter spatial and subsecond temporal resolution. This is orders of magnitude better than previous techniques.

The focus of these studies over the last year has been the continued evaluation of motor and visual processing in the brain as well as new efforts in pain, taste and auditory stimulation. A great deal of effort has been devoted to a better understanding of the physiological nature of the signals observed as well as the source of background "noise." The motor studies have progressed to the development of new stimuli protocols and the documentation of brain responses to real and imagined actions. The differences in brain images acquired during actual performance of a task versus imagined performance of the same task provide important information on the processing of information within the brain.

The pain studies have revealed very high resolution studies (200 micron, in-plane resolution) of the sensory cortex location for a painful heat stimulus on the fingers. Hopefully this type of information will provide insight into the complex processing of pain in the human brain.

The large effort in evaluating the nature of the background "noise" and physiological origin of the signals observed has revealed that a significant source of signal is from the large draining veins of the cortex. However, this effect can be limited by appropriate choice of experimental conditions. In addition, the background noise is largely due to flow and motion irregularities which cause a random variation in the signal intensity. New methods to suppress this noise source and better localize the metabolic responses are now in development.

ANNUAL REPORT OF THE CARDIOLOGY BRANCH
National Heart, Lung, and Blood Institute
October 1, 1992 through September 30, 1993

The experimental interests of the Cardiology Branch have evolved over the past year as a result of our developing a section on molecular biology, while maintaining our collaborations in the area of molecular genetics, and continuing to integrate these basic disciplines with our longstanding strengths in clinical and basic physiologic investigations.

VASCULAR BIOLOGY AND CORONARY RESTENOSIS

Vascular response to injury: Increasing evidence implicates the smooth muscle cell (SMC) in the process of atherogenesis. Thus, it appears that SMCs are activated in response to injury, causing their proliferation and migration from the media to the intima. A similar but markedly accelerated process causes restenosis following coronary angioplasty in 25-50% of pts weeks or months later. Given this pathophysiologic mechanism, it would appear that the problem of controlling restenosis may lie in the control of the SMC response to injury. The focus of the research efforts of this section is twofold: 1) to derive insight into the basic mechanisms responsible for the restenosis process and, 2) to develop novel molecular-based strategies to prevent coronary restenosis after angioplasty.

Basic mechanisms: To determine whether an abnormality in the cellular mechanisms that regulate SMC proliferation may contribute to restenosis, we studied the tumor suppressor gene *p53* and its protein product in atherectomy specimens of restenosis lesions from 60 pts. *p53* inhibits cell cycle progression; its dysfunction occurs commonly in human cancers. Dysfunction is usually caused by a mutated gene expressing a mutated, more stable product, causing *p53* accumulation and immunopositivity (im-pos). (Tissues with normal *p53* are im-neg). We found *p53* im-pos SMCs in almost 40% of the lesions. Genomic DNA sequencing of im-pos lesions revealed wild-type *p53*. Since elevated *p53* is not due to gene mutation, we initiated studies to determine whether viral oncoproteins are present that bind to and inactivate *p53*, thereby playing a causative role. Because prior evidence implies a causal role for human cytomegalovirus (HCMV) in atherogenesis, we examined HCMV's role in restenosis. We found that 1) a correlation exists between elevated *p53* and HCMV genome in restenosis lesions, 2) HCMV infection elevates *p53* levels in SMCs, 3) HCMV IE2 inhibits *p53* transcriptional activity. Thus, HCMV may play a causal role in restenosis, which may be mediated by inhibiting the suppressor effects of *p53*.

The *ras* proto-oncogene has been implicated in signal transduction, although its role in SMCs is unknown. We employed a dominant negative (*rasDN*) gene whose expression is known to block *ras*-dependent pathways. Using a transient transfection assay, A10 rat aortic SMCs were co-transfected with a plasmid encoding the interleukin-2 receptor (pCMV-IL2R;1 μ g) acting as a cell surface marker, and either the *rasDN* plasmid (pCMV-*rasDN*;5 μ g) or a control plasmid (pCMV;5 μ g). We found that the expression of the dominant negative *ras* gene inhibits signal transduction from a variety of important SMC mitogens including PDGF, EGF and FGF. We are attempting to use the expression of this dominant negative *ras* gene and other molecular techniques to dissect the various mitogenic and migratory pathways that contribute to the abnormal SMC proliferation that is evident in restenosis.

Molecular-based therapeutic strategies. Last year we undertook studies of antisense approaches to inhibit SMC proliferation, and the use of genetically engineered recombinant chimeric toxins to kill activated SMCs. These approaches worked in vitro, and the results have stimulated other laboratories test in vivo potential in animal models of vascular injury. This year we focused on a replication deficient recombinant adenovirus to deliver a variety of therapeutic gene products to the vessel wall. We demonstrated that adenovirus can efficiently and selectively transfer gene to SMCs in areas of vascular injury. We are presently attempting to inhibit restenosis by designing adenoviruses that encode antiproliferative gene products. Our preliminary results indicate that the expression of one such adenovirus-delivered gene does inhibit SMC proliferation in vitro--we are now exploring the possibility that this strategy may provide an intravascular gene-therapy-based treatment for restenosis and, ultimately, atherosclerosis.

ANGIOGENESIS AND ENHANCEMENT OF CORONARY COLLATERAL DEVELOPMENT

CAD pts who develop ischemic symptoms refractory to pharmacologic therapy and who are not candidates for bypass surgery pose a major therapeutic problem. Several years ago we postulated that one approach to this dilemma would be to devise a means of enhancing collateral growth, and thereby collateral flow, to ischemic regions of the myocardium.

Prior attempts to substantially increase collateral flow by pharmacologically means have failed. However, several polypeptides with the potential to cause angiogenesis have become available for use. We therefore undertook a series of studies to determine whether use of such peptide growth factors might enhance myocardial collateral blood flow. Our ultimate goal would be to utilize these agent(s) to facilitate myocardial revascularization in pts with coronary heart disease.

Ameroid constrictors were used to progressively occlude the left circumflex (LCX) coronary artery of dogs. Some degree of intrinsic collateral growth develops in response to this stimulus. We determined whether basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF) could augment the intrinsic collateral development. In our original experiments, we found that 20 days of *intracoronary* administration of bFGF (110 μ g/d) or VEGF (45 μ g/d) increased collateral blood flow by 40% after approximately one month. In a second experiment we found that it was not necessary to give the peptide directly into the coronary vessels; 20 days of *systemic* administration of bFGF (1.74 mg/d) also accelerated collateral development substantially. To determine whether the difference between the control and treated groups seen over one month persisted, we performed a longer "crossover" study, in which collateral flow was assessed over a period of two months. Again bFGF, given systemically (1.74 mg/d), enhanced collateral flow, and the difference was maintained for the entire study duration. Interestingly, there was no difference between dogs receiving bFGF for 5 weeks and those receiving it for 9 weeks. In fact, the major improvement was limited to the period of 10-17 days after placement of the constrictor, presumably the time ischemia was developing. A fourth study was therefore carried out to determine whether one week (instead of one month) of treatment could result in the same

increase in collateral flow. The results of this experiment were dramatic and definitive; this short period of treatment increased flow by over 65%. Thus, more prolonged exposure to bFGF appears to be unnecessary. The results of this entire series of experiments may have important clinical implications: collateral blood flow can be enhanced pharmacologically by bFGF, and only a brief period of drug administration is necessary to bring about this effect, thereby possibly obviating the potential deleterious side effects attendant upon more prolonged administration in pts.

We plan further studies in which the effect of bFGF will be assessed on a long term (one year or more) basis, studies to determine whether even shorter intervals of administration are effective, and studies in which the peptide will be delivered via gene transfer.

HYPERTROPHIC CARDIOMYOPATHY (HCM)

HCM is an inherited heart disease that is the most common cause of sudden death (SD) in otherwise healthy young individuals. Pts with hypertrophic cardiomyopathy (HCM) frequently have severe symptoms and clinically important arrhythmias, and are prone to sudden death (SD) and syncope. Our studies in HCM pts are directed at: 1) improving risk stratification of pts, 2) elucidation of mechanisms of SD and syncope, 3) relieving symptoms in pts with obstructive HCM, 4) developing effective antiarrhythmic therapy, and 5) understanding the genetics and its pathophysiology at a molecular level.

Pacemaker therapy as alternative to cardiac surgery in pts with obstructive HCM:

We have shown in now more than 150 pts that dual chamber pacing is effective in relieving drug-refractory symptoms and in reducing LV outflow obstruction. The advantage of this therapy over traditional LV myectomy or mitral valve replacement is its low cost and lower mortality and morbidity. An important finding has been that chronic pacing alters electrical and hemodynamic cardiac properties that are evident even when pacing is discontinued. We intend to investigate the molecular basis of this further.

Sudden cardiac death and syncope: Although arrhythmias are the most common cause of SD in adults with HCM, we have determined that myocardial ischemia, often silent, is the most common cause of syncope and cardiac arrest in young HCM pts.

Genotyping: With the report that β MHC gene is responsible for HCM in some kindreds, attempts have been made to identify HCM kindreds with distinct β MHC gene mutations. We have identified 12 distinct mutations in the β -myosin heavy chain (β MHC) gene, located on chromosome 14, in 16 unrelated kindreds, and estimate that about 20% of HCM is caused by mutations in this gene. We have excluded the β MHC gene locus, and two other novel loci, in 4 large families. These large families are being used in gene mapping studies to identify new loci and thus work towards identifying other genes responsible for HCM.

Correlation of genotype and phenotype: Molecular markers have been used to identify individuals without LV hypertrophy but with symptoms and arrhythmias caused by β MHC gene mutations. Detailed clinical evaluations indicate that the natural history of the disease is in part determined by the specific mutation.

Expression of the β MHC gene and the pathophysiology of the protein: We have demonstrated that mutant messenger RNA and beta myosin are expressed in skeletal muscle

of affected pts. We also showed that skeletal muscle myofibers containing mutant β -myosin have abnormal function in an in-vitro motility assay. Histochemical analyses demonstrated that 60% of pts with β MHC gene mutations have a skeletal myopathy--specifically, "central core disease", a very rare, nonprogressive myopathy characterized by loss of mitochondria from the center of some of the slow myofibers. Central core disease was present in some children and adults with mutations but without cardiac hypertrophy. This is consistent with the idea that hypertrophy is a secondary phenomenon.

ENDOTHELIAL FUNCTION: ITS CONTRIBUTION TO CORONARY VASCULAR TONE, PERIPHERAL VASCULAR TONE, AND PLATELET FUNCTION IN MAN AND ITS CONTRIBUTION TO MICROVASCULAR DYSFUNCTION

The endothelium of blood vessels secretes substances that dilate vascular smooth muscle and inhibit platelet aggregation. This year we have continued our studies designed to elucidate the normal mechanisms by which the endothelium modulates coronary and systemic vascular tone and influences platelet function, and how aberrations of its normal function may influence and contribute to the development of various disease states.

Endothelial Function in Hypertension and Hypercholesterolemia

We have shown that pts with hypertension and pts with hypercholesterolemia have impaired endothelium-dependent vascular responses to acetylcholine (ACH), and that this abnormality is related to a defect in the nitric oxide system. Previous studies from our laboratory had shown that L-arginine, the precursor of nitric oxide, augments the response to ACH in normal humans, indicating that the availability of nitric oxide substrate is rate-limiting for endothelium-dependent vasodilation. We had also shown that this mechanism fails to operate in hypertensive pts. Over the last year, we extended these observations to hypercholesterolemic pts, demonstrating that the impaired vascular responses to ACH are not significantly modified by the administration of L-arginine. This indicates that the impaired endothelial function of these pts is not related to decreased availability of the precursor for nitric oxide formation.

Another important issue was whether the abnormal endothelium-dependent vascular responses of hypertensive and hypercholesterolemic pts were related to a specific defect of the muscarinic receptor, since previous investigations had invariably used muscarinic agents. To address this issue, we studied the response of the forearm vasculature to substance P, an endothelium-dependent vasodilator that acts through a different receptor on the endothelial cell (although through the same signal transduction pathway). The results showed that pts with hypertension and pts with hypercholesterolemia have a depressed response to substance P when compared to normal controls. Hence, our findings demonstrate that the endothelial dysfunction in hypertension and in hypercholesterolemia is not isolated to the muscarinic receptor, thus implying a broader abnormality of the vascular endothelium. We are also attempting to determine whether hypercholesterolemic pts have impairment of more than one signal transduction pathway by comparing the forearm vasodilator response to the endothelium-dependent vasodilator ACH to the endothelium-dependent vasodilator bradykinin, which employs a different signal transductional pathway.

Role of estrogen: Estrogen replacement therapy has been associated with reduction of cardiovascular events in postmenopausal women, although the mechanism of benefit is unknown. Recent in vitro and in vivo animal data suggest that estrogen may protect against vasoconstriction by means of both endothelium-dependent and endothelium-independent mechanisms. Preliminary results of our current studies performed in 35 postmenopausal women not on estrogen replacement indicate that the acute intraarterial administration of estradiol into the forearm augments the vasodilator response to the endothelium-dependent agonist ACH, but not the endothelium-independent agonist sodium nitroprusside. Estradiol levels achieved in the venous outflow of the forearm were comparable to those measured in midcycle of reproductive-age women. Of 16 postmenopausal women undergoing cardiac catheterization, similar estradiol-induced enhancement of the coronary vasodilator response to the endothelium-dependent agonist ACH was observed.

Preliminary results of the effect of chronic (3 weeks) administration of estradiol by a patch preparation has failed to show an enhancement of the endothelium-dependent forearm vasodilator response to ACH. A likely explanation for this finding relates to the plasma levels of estradiol achieved with the chronic patch preparation, which were approximately 1/3 of that achieved with the acute infusion. Repeat infusion of estradiol to increase estradiol levels comparable to that achieved during the acute infusion study restored the enhancement of the vasodilator response to ACH to a degree similar to that observed during the initial acute infusion of estradiol.

Role of oxidized LDL: Studies performed by our group and others have shown impaired vasodilator responses to the endothelium-dependent agonist ACH in the coronary and systemic circulation of hypercholesterolemic pts, even in the absence of angiographically apparent coronary artery disease. Animal studies have shown that oxidized LDL may be responsible for impairment of nitric oxide production by the endothelium. Our current study seeks to determine the pathophysiologic mechanisms underlying this impairment, and to determine whether treatment with antioxidant vitamins beta carotene, C, and E can improve the vascular responses to endothelium-dependent vasodilators. The responses of hypercholesterolemic pts are compared to age and gender matched normal individuals. Antibody titers to oxidized low density lipoprotein are being measured to assess their predictive value regarding impaired endothelium-dependent vasodilation in hypercholesterolemic pts. Hypercholesterolemic pts are then being treated for 4 weeks with beta carotene, vitamin C, and vitamin E, with a repeat of the forearm flow studies to determine whether the administration of vitamin antioxidants can improve endothelium-dependent vasodilation. The effect of antioxidant therapy on platelet aggregability and on the susceptibility of low density lipoprotein to oxidative stress is also being assessed.

Endothelial contribution to exercise-induced vasodilation: LNMMA, an inhibitor of nitric oxide release, produced a 25% reduction in forearm vasodilation that accompanies exercise, suggesting that nitric oxide release contributes to at least 25% of forearm vasodilation that occurs with exercise.

Coronary microvascular dysfunction: Approximately 40% of pts with chest pain and normal coronary arteries have endothelial dysfunction of the coronary micro vasculature demonstrated as a reduced response to intracoronary ACH. These pts also have a depressed vasodilator response to atrial pacing, suggesting that microvascular endothelial dysfunction

contributes to the reduced dilator response in microvascular angina. Approximately 40% of pts with hypertension and normal epicardial coronary arteries also have endothelial dysfunction of the coronary microvasculature.

To investigate whether endothelial dysfunction of the coronary microvasculature in pts with chest pain and normal coronary arteries is localized to the coronary vascular endothelium, or is a generalized disorder of endothelial function, we studied the response of both the coronary and femoral vasculature to ACH. There was a correlation between cardiac and peripheral vascular endothelium-dependent vasodilation, such that pts with depressed coronary microvascular dilator response to ACH also had depressed responses in the femoral circulation and vice versa.

To further investigate the causes underlying the endothelial dysfunction present in the peripheral microvasculature, we studied the role of coronary risk factors in the development of endothelial dysfunction. There was a correlation between the number of risk factors (the presence of atherosclerosis, age > 60, cholesterol > 270 mg/dl, HDL < 35 mg/dl, hypertension, diabetes, heart failure, and smoking) and the response to ACH, suggesting that peripheral microvascular endothelial dysfunction is present in pts with multiple coronary risk factors.

Administration of intracoronary L-arginine, the precursor of nitric oxide, improved the coronary microvascular responses to ACH; this effect was most evident in pts with a reduced response to ACH. Thus, the reduced availability of substrate for nitric oxide production may account for, at least in part, the endothelial dysfunction observed in pts with microvascular angina.

AORTIC REGURGITATION

Transesophageal Dobutamine Echocardiography in Aortic Regurgitation: Both left ventricular dimensions and systolic function at rest have been shown to be useful tools for the prognostic assessment of pts with aortic regurgitation (AR). However, determination of these indices at one point in time does not precisely identify the pts who are more likely to show progressive left ventricular dilatation or deterioration in systolic function during follow-up, and who thereby would need operative intervention. Dobutamine stress echocardiography has been shown to be a useful method for the clinical assessment of myocardial contractility of pts with coronary artery disease. We have therefore begun to use transesophageal dobutamine echocardiography to determine myocardial inotropic reserve in pts with AR who are asymptomatic and have preserved resting systolic function at rest.

Our results show that the myocardial inotropic reserve is independent of either left ventricular dimensions or systolic function at rest. It remains to be determined whether this index of myocardial contractile state can predict the development of left ventricular dysfunction, progression of left ventricular dilatation, or appearance of symptoms during follow-up.

CORONARY ARTERY DISEASE

Identification of Viable Myocardium in Pts with LV Dysfunction: In many pts with chronic coronary artery disease, impaired LV function at rest represents chronic hypoperfusion, termed hibernating myocardium. Identification of pts with such potentially reversible LV

dysfunction has been problematic, as regional dysfunction arising from hibernating but viable myocardium may be clinically indistinguishable from that arising from infarcted myocardium.

Over the past two years, we have demonstrated that thallium reinjection after a 3-4 hour redistribution imaging may identify such pts with viable but dysfunctional myocardium. The similarities between published results of thallium reinjection and positron emission tomography (PET) for predicting recovery of function after revascularization prompted us to perform a comparative study of the two imaging techniques in the same pts with chronic CAD and LV dysfunction. In severe irreversible thallium defects in which viability would be a clinically important issue, the results of thallium reinjection were comparable to PET. However, PET is very expensive, requires cyclotron technology, and is not readily available. On the other hand, thallium is widely available for clinical use and provides a less expensive alternative to PET. Hence, in the current era of cost containment, it would seem prudent for nuclear cardiology laboratories to use thallium for assessing myocardial viability.

Do persistent regional wall motion abnormalities following exercise represent stunned myocardium? Whether stunned myocardium occurs after exercise-induced myocardial ischemia remains controversial. We studied pts with chronic CAD, all of whom had abnormal LV ejection fraction (EF) response from rest to exercise as assessed by radionuclide angiography (RNA). A subgroup of pts also underwent ^{82}Rb myocardial blood flow (MBF) study using PET. In 88% of pts, regions with abnormal exercise-induced rEF had persistent or worsening of rEF at 15 min post-exercise and in 65% of pts the abnormal response persisted at 30 min post-exercise. Among the subgroup of pts with both rEF and MBF studies, regions demonstrating decreased rEF during exercise and 15 min post-exercise were associated with lower MBF compared to normal regions. At 30 min post-exercise, MBF continued to track rEF; 50% of the regions demonstrating concordant improvement in rEF and MBF and 50% having persistent decrease in rEF and MBF. The finding of decreased MBF in regions with decreased rEF post-exercise does not conform to the classic definition of stunned myocardium. Although the mechanisms responsible for persistent post-exercise decrease in MBF are not known, any one of the ascribed causes of coronary artery vasoconstriction during exercise may be operative: sympathetic nervous system activation, endothelial dysfunction, or hydrodynamic constriction.

Annual Report of the Laboratory of Cell Biology
National Heart, Lung, and Blood Institute
October 1, 1992 to September 30, 1993

The Laboratory organization remains as it was with the following tenured staff: Cellular Biochemistry and Ultrastructure Section: Edward D. Korn, Chief, M. Blair Bowers, J. Knutson, R. Chen and H. Brzeska-Bzdega (approved this year for conversion to a permanent position); Molecular Cell Biology Section: J. A. Hammer, III, Chief; Cellular Physiology Section: E. Eisenberg, Chief, L. E. Greene; Membrane Enzymology Section: R. W. Hendler, Chief; Pulmonary and Cardiac Assist Devices Section: T. Kolobow, Chief. The Laboratory occupies approximately 9800 square feet in Building 3 and 2000 square feet in Building 10 with the use of animal surgery space in Building 14E. Major research interests continue to be: (1) the structure and function of non-muscle myosins, (2) the regulation of actin polymerization, (3) the structure and function of the 70-kDa heat shock proteins, (4) bioenergetics, (5) biological applications of fluorescence spectroscopy, (6) respiratory assist devices, and (7) membrane flow in eukaryotic cells. The following summary of this year's accomplishments is arranged by scientific topics, not organizational Sections.

Myosin Superfamilies in Amoebae: Previous work by Dr. Korn and his colleagues described two families of myosin in *Acanthamoeba castellanii*, one myosin II isoform (a classical filamentous myosin with two heavy chains and two pairs of light chains) and 3 non-filamentous myosin I isoforms (monomers with a single heavy chain). Dr. Hammer and his collaborators had described a third family, high-molecular-weight myosin I, which thus have highly purified and shown to have actin-activated Mg^{2+} -ATPase activity and a single heavy chain with a long, flexible tail.

One myosin II and 6 myosin I isoforms (which fall into 2 subfamilies) have been identified, at the protein and/or DNA level, in *Dictyostelium* with DNA evidence for 6 other isoforms. Hammer's group has now cloned and begun sequencing 2 of these and is cloning the other 4.

Conformational Regulation of *Acanthamoeba* Myosin II Activity: Previous studies by Dr. Korn had established that the actin-activated Mg^{2+} -ATPase activity in the N-terminal, globular head of filamentous myosin II is regulated by phosphorylation of 3 serines at the C-terminal tip of the coiled-coil helical rod-like tail. Experimental data supported a mechanism based on a filament structure in which the C-terminal tails clustered around a hinge region in the heavy chains. When the C-terminal tails were phosphorylated it caused the hinge to be much more flexible and this dramatic decrease in hinge stiffness in the rod domain could be geometrically transmitted to the head domain and affect its interaction with F-actin. However, data showing that the sites in the heads of monomeric myosin II that were cleaved by endoprotease Arc-C were regulated by phosphorylation at the tip of the tail provided strong evidence for head-tail interaction in monomers. Additional evidence for this has now been obtained. Differential scanning calorimetric studies show highly cooperative interactions between the head and tail domains of monomeric myosin II such that

endothermic unfolding occurs with a single, narrow isotherm at 42° C compared to a broad transition between 38 and 65° C for muscle myosin II. Binding of MgATP to the head domain stabilizes the head and separates its unfolding isotherm from the tail transition. MgATP bound to the head also accelerates the rate of cleavage at the tip of the tails of filamentous myosin II by papain.

Regulation of *Acanthamoeba* Myosin I Heavy Chain Kinase: Dr. Korn's group had shown that the actin-activated Mg^{2+} -ATPase activity of myosin I is regulated by phosphorylation of a single site in the head domain by myosin I heavy chain kinase. The kinase is activated by autophosphorylation and the rate of autophosphorylation is greatly accelerated by acidic phospholipids and plasma membranes. Activation of myosin I heavy chain kinase by plasma membranes *in vitro*, which presumably models the situation *in vivo* has been studied in more detail. It was found that, although the membranes do accelerate the rate of autophosphorylation of the kinase (as previously reported), full activation of kinase activity occurs before significant phosphorylation has occurred. Thus, a conformational change induced by binding to the membranes is sufficient to activate kinase activity.

Biologic Functions of the Myosin I Family: In previous studies, Dr. Korn's group had demonstrated differential localization of the 3 *Acanthamoeba* myosin I isoforms: IA at the actin-rich cell cortex and associated with small cytoplasmic vesicles; IB at the plasma membrane (enriched at pseudopodal and phagocytic cup membranes) and the membranes of large cytoplasmic vesicles; IC at the plasma membrane (different distribution than IB), large cytoplasmic vesicle membranes and the contractile vacuole membrane. This year, a specific myosin IC antibody that inhibits phosphorylation of myosin IC by kinase and inhibits actin-activated Mg^{2+} -ATPase activity of myosin IC *in vitro* was introduced into viable cells. When exposed to hypotonic media, the cells swelled and lysed because contractile vacuole function was arrested. This is the first evidence for a specific role for a membrane-associated myosin I.

Dr. Hammer's group had previously shown that 5 of the 6 sequenced *Dictyostelium* myosins I fall into 2 subfamilies; myoB, myoC and myoD are structurally similar to the *Acanthamoeba* myosin I family and are constitutively expressed while myoA and myoE, which are up-regulated by chemotactic aggregation, have shorter heavy chains that lack the actin-binding region but retain the membrane-binding region of the other myosin I isoforms. MyoB and myoD have similar cellular localizations to those of *Acanthamoeba* myosin IB. MyoB⁻ null cells had been shown previously by Hammer to have reduced rate of cell migration, chemotaxis and phagocytosis but a double mutant, MyoB⁻/myoD AS (antisense) had little additional impairment. Hammer has now found that the triple mutant myoB⁻/myoD AS/myoC AS is dramatically impaired in chemotactic aggregation and development and grows slowly. These important functional assays are consistent with the localization of this myosin I subfamily and with the functions of *Acanthamoeba* myosin IB inferred from its localization.

Regulation of Actin Polymerization: Actobindin, a 9-kDa protein from *Acanthamoeba*, had previously been shown to be a potent inhibitor of nucleation of actin filaments but

not of filament elongation and to bind 2 actin monomers. Evidence has now been obtained by Dr. Korn that actobindin has a higher affinity for a covalently cross-linked actin dimer than for 2 monomers, that it catalytically generates a greater than stoichiometric concentration of nucleation-incompetent, elongation-competent dimers from monomers and, using the rate constants for dimer formation and dimer addition to filament ends, that the kinetics of actin polymerization can be modeled over a wide range of actin and actobindin concentrations. These results provide strong evidence that actobindin function *in vivo* to inhibit random formation of actin filaments while facilitating elongation of pre-existing uncapped filaments. Actobindin has been immunolocalized to newly forming pseudopods where just such properties would seem to be useful.

Profilin I and II have been shown to be differentially localized in *Acanthamoeba*: profilin I mostly in the cortical regions associated with G-actin (suggesting it functions mostly to sequester actin monomers) and profilin II at the plasma membrane (suggesting it may function mostly to regulate metabolism of membrane-associated PIP₂).

Muscle and amoeba F-actin have been found to behave differently when added to amoeba extracts under polymerizing conditions: muscle F-actin depolymerizes more rapidly than in buffer alone and amoeba F-actin is much more stable than in buffer. Thus, there appear to be stabilizing and destabilizing proteins present that interact differently with the 2 actins. This raises questions about the common use of muscle F-actin as an indicator of the behavior of cell actin in non-muscle cells.

Uncoating ATPase and 70-kDa Heat Shock Proteins: Drs. Greene and Eisenberg have been studying the bovine brain uncoating ATPase, a 70-kDa heat shock protein that uncoats clathrin-coated vesicles with accompanying hydrolysis of ATP. New results this year include the discovery of a 100-kDa protein that acts as a required cofactor for both the uncoating and the ATPase activity at a saturating molar ratio of 1:10 relative to both clathrin and the uncoating ATPase. The 100-kDa protein was discovered during the purification of AP2, one of several assembly proteins that individually induce the assembly of clathrin into baskets.

During these studies a new, 20-kDa assembly protein was discovered and shown to induce the formation of normal-appearing clathrin baskets. This is particularly interesting because all previous assembly proteins are much larger, about 100 kDa - 300 kDa and in some cases consist of multisubunits. Another protein factor has been partially purified that causes an initial burst of ATPase activity 25-fold greater than the steady state when added to uncoating ATPase.

Previous studies on the role of ATP in the uncoating reaction have been extended Greene and Eisenberg's group to show that dissociation of clathrin and other bound peptides from the uncoating ATPase is much faster in the presence of ATP than in the presence of bound ADP and Pi. This suggests that ATP binding rather than ATP hydrolysis may be responsible for clathrin dissociation. Surprisingly, nucleotide-free uncoating ATPase acts like ATPase with bound ATP with respect to clathrin binding

but like ATPase with bound ADP with respect to binding of peptides.

Bioenergetics: Dr. Hendler is studying the kinetics of the transfer of 4 electrons from cytochrome c to the iron (a and a₃) and copper (CuA and CuB) centers of cytochrome aa₃ using a new ultrarapid spectrometer designed and built in his laboratory and analyzing the spectral data by newly developed methods based on the generalized pseudoinverse of linear algebra. The spectral behavior of cytochromes a and a₃ have been separated for the first time. With the resting enzyme, the first electron is passed rapidly (4.6 ms) and is shared equally between heme a and CuA. The second electron passes slowly (25 min) and the 2 electrons are shared equally by all 4 centers. In the presence of oxygen, the resting enzyme is activated, the transfer of electrons from a to a₃ is greatly accelerated and 4 electrons are transferred, reducing all 4 centers.

The laser-activated photocycle of bacteriorhodopsin, another biologically important energy-transducing pump, has been studied by similar methods. The existence of two different intermediates has been confirmed: the lower the light intensity the greater the ratio of the fast cycle intermediate (M_f) to the slow cycle intermediate (M_s). The new discovery was that these intermediates represent two different photocycles, one dominating at low light and the other at high light intensity. Preliminary evidence suggests that different associations with membrane phospholipids may be the mechanism by which a single protein is able to catalyze 2 different photocycles.

Biological Application of Fluorescence Spectroscopy: Dr. Knutson has used the laser-based, time-resolved fluorescence spectroscopy system he constructed and continues to develop to initiate studies on folding and dynamics of several proteins. Effort this year was focussed mainly on the interaction of DNA with proteins that regulate transcription, specifically the interaction of oct-pou which can bind to two different classes of DNA and increase the rate of transcription 100-fold. The folding of the oct-pou protein was shown to depend on the sequence of the DNA with which it interacted. Similar studies have begun with other DNA-binding proteins including TFIIIA, heat shock factor and HIV integrase. Although all of these studies are of interest in their own right, the principal effort of this group is to develop and demonstrate the utility of this powerful methodology so that it will be widely used in studies of protein conformation.

Dr. Chen has used fluorescence spectroscopy to study the interaction of heparin and the anti-tumor agent Suramin with basic fibroblast growth factor (bFGF); the binding of bFGF to its receptor is dependent on its prior binding to heparin and is inhibited by Suramin, even in the presence of heparin. In addition to the previous observation that heparin quenches the fluorescence of the single tryptophan, the new data show 40% quenching of the fluorescence of the 11 tryptosines. Thus, the conformation of extensive regions of bFGF is influenced by heparin binding.

Some of the basic concepts of fluorescence of proteins and peptides are being studied and challenged by Chen. Recent data indicate that, contrary to accepted

dogma for all fluorescence systems, the lifetime and quantum yield of tryptophan fluorescence of more than 20 proteins are not directly proportional. Fluorescence measurements have also shown that chloride ions enhance the rate of protonation of excited-state serotonin and related 5-hydroxyindoles. This may provide a sensitive assay for chloride ions and also for the interaction of chloride ions with biomolecules.

Respiratory Assist Devices: Dr. Kolobow has found previously that the mechanical ventilation systems often used to alleviate severe forms of acute respiratory failure in patients can themselves induce acute respiratory failure in healthy lungs. He is developing systems to provide gentler mechanical ventilation to reduce or eliminate lung damage. Kolobow has developed intratracheal pulmonary ventilation systems using endotracheal tubes of his own design and fabrication that cause minimal or no lesions on laryngeal and tracheal structures of rabbits and sheeps after 24 hours of continued use. These devices show great clinical potential.

ANNUAL REPORT OF
THE LABORATORY OF CELLULAR METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE

October 1, 1992 through September 30, 1993

Research in the Laboratory of Cellular Metabolism has for several years concentrated on guanine nucleotide-binding proteins that are critical control elements and signal transducers in cellular responses to the extracellular environment. Many of these are substrates for bacterial toxins that catalyze the ADP-ribosylation of specific amino acids and thereby alter protein activity. The properties and actions of some of these toxins have also been investigated, along with continuing attempts to identify and characterize analogous ADP-ribosyltransferases in animal cells, as well as the enzymes that remove the ADP-ribose and the proteins that are substrates in these kinds of potentially regulatory ADP-ribosylation cycles. Long term studies of specific cyclic nucleotide phosphodiesterases (PDEs) are now yielding considerable new information about a family of these enzymes that are important sites of hormonal effects in many cells and are targets of several new drugs. Recently, much of our effort has been focussed on the 20-kDa ADP-ribosylation factors (ARFs) that are involved in intracellular vesicular transport to Golgi membranes and probably in other locations. This has included cloning and expression to evaluate structure-function relationships and effects of post-translational modification as well as attempts to define interactions of individual specific ARFs with Golgi in vitro.

1. Cyclic Nucleotide Phosphodiesterase

By degrading cAMP and cGMP, PDEs are critical determinants in the regulation of intracellular cyclic nucleotide concentrations. Five different PDE gene families have been identified and characterized. They differ in structural and biochemical properties, substrate affinities, sensitivities to specific inhibitors and regulatory properties. For the past several years, our laboratory has focused on the "so-called" Type III cGMP-inhibited PDE gene family. Type III (or cGI) PDEs are characterized by high affinity ($K_m < 1 \mu M$) for cAMP and cGMP, selective and specific inhibition by a number of positive inotropic agents and rapid activation by insulin and hormones that increase cAMP in adipocytes, hepatocytes, and platelets. They have been implicated in regulation of several important physiological processes, e.g., lipolysis, myocardial contractility, smooth muscle relaxation, platelet aggregation. We have extensively studied the rat adipocyte cGI PDE, which is phosphorylated and activated by insulin. Its activation is important in the antilipolytic action of insulin.

We previously cloned cDNAs for three cGI PDEs - RcGIP1 and RcGIP2 from rat adipose tissue and HCAR from human cardiac tissue cDNA libraries. This year we cloned a cDNA (HFAT) for a human adipose tissue cGI PDE and extended the 5' sequence of RcGIP2, which is still 100-200 bp from the estimated translation start site. These cGI PDEs represent two distinct subfamilies of the cGI PDE gene family, products of two different but related genes.

Deduced amino acid sequences of four cGI PDEs exhibit a common chimera-like structural pattern, with the catalytic domain (conserved among all mammalian PDEs) in the C-terminal portion followed by a hydrophilic domain. The N-terminal regulatory region contains hydrophobic putative membrane association domains and several consensus cAMP-dependent protein kinase

phosphorylation sites, consistent with the known phosphorylation of several cGI PDEs by cAMP-dependent protein kinase. RcGIP1 is more closely related to HFAT than to RcGIP2, which is more closely related to HCAR than to RcGIP1. Within the conserved catalytic domains of the four cGI PDEs is an insertion of 44 amino acids, which does not align with other PDE families. The sequence of the insertion is very similar in RcGIP1 and HFAT, and differs from that in RcGIP2 and HCAR, which are very similar to each other. This insertion may not only differentiate cGI PDEs from other PDE Types but may also be important in identification of individual members or subfamilies within the Type III gene family. RcGIP1 and HFAT have little similarity to RcGIP2 and HCAR in the regulatory domains. All four have very similar hydropathy plots, suggesting similar structural and functional domains.

A single ~5.3-kb mRNA for RcGIP1 is highly expressed in rat adipocytes and rat testis. Multiple mRNAs for RcGIP2 found in rat adipose tissue (not isolated fat cells), heart, aorta, and lung. RcGIP1 mRNA is markedly increased during differentiation of murine 3T3-L1 adipocytes. (Our earlier studies had indicated that in 3T3-L1 adipocytes, a particulate cGI PDE appears, or markedly increases, during differentiation.) Stable expression of RcGIP1 in NIH-3006 fibroblasts, which over-express the human insulin receptor, yielded several transfectants with PDE activity >100-times that in cells transfected with plasmid alone. The expressed cGI PDE was of the predicted size (~125 kDa) on SDS-PAGE, reacted with anti-cGI PDE antibodies on Western immunoblots, and was inhibited by cilostamide and cGMP. Incubation of these cells with insulin increased PDE activity of total homogenates of several clonal lines by 30-100%.

All our results suggest that RcGIP1 and RcGIP2 represent two distinct cGI PDE subfamilies. RcGIP1 represents the rat adipocyte hormone-sensitive cGI PDE; RcGIP2, perhaps a cardiovascular-type. Among these four cGI PDEs there is more conservation between rat and human members of the same subfamily than between different subfamilies in the same species, rat or human. As yet, we have not identified other cGI PDE subfamilies. Using degenerate oligonucleotides 5' and 3' to the additional region in the conserved cGI PDE catalytic domain, we have attempted to amplify by RT-PCR unique cGI PDE cDNAs from human aortic, hepatic, and HEPG2 hepatoma mRNA and cDNA, but we have only generated and subcloned cDNA fragments corresponding to HCAR or HFAT.

In the past year, to initiate structure/function studies, we expressed "full-length (monomer M_r ~125 kDa)" and "truncated" (monomer M_r ~54 kDa)" recombinant HCAR cGI PDEs in baculovirus-infected Sf9 cells. Both exhibit K_m s for cAMP as substrate and sensitivities to inhibition by cilostamide similar to those of cGI PDE in human cardiac microsomes. The three cGI PDEs are not sensitive to inhibition by Rolipram, a specific inhibitor of Type IV PDEs. Activity of the truncated form, which contains the catalytic domain but lacks the N-terminal region hydrophobic domains, was found predominantly in Sf9 cytosol (100,000 g supernatant) whereas the full-length form was predominantly particulate. The "full-length" RcGIP1, expressed in Sf9 cells, was also found in association with particulate fractions and exhibited K_m values for cAMP and sensitivity to cilostamide which were similar to those of a rat adipocyte particulate cGI PDE. Thus, the mechanism for association of cGI PDEs with intracellular membranes, in adipocytes for example, might be successfully elucidated with expression of appropriate recombinant forms in Sf9 cells.

We are attempting to define the limits of the cGI PDE catalytic domain by expressing constructs of different sizes as fusion proteins in *E. coli*. Catalytic activity of RcGIP1 and HCAR and sensitivity to inhibition by cilostamide resides in a C-terminal region of ~500 amino acids, which contains

the domain (~250 amino acids) conserved among all mammalian PDEs. The conserved domain plus additional (perhaps ~100) upstream amino acids, but no additional downstream sequences, may be required for catalytic activity. The availability of "full-length" and "truncated" RcGIP1 and HCAR recombinant cGI PDEs will allow for a detailed comparison of the properties of two different cGI PDE subfamilies. This may have important implications for design of selective inhibitors which could be effective therapeutic agents for certain types of cardiac failure.

2. ADP-ribosylation Factors (ARF): 20-kDa Guanine Nucleotide-Binding Proteins

ARF was first identified in Gilman's laboratory as a GTP-binding protein required for cholera toxin-catalyzed ADP-ribosylation of purified $G_{\alpha s}$. We later showed that ARF enhances all toxin catalytic activities, independent of ADP ribose acceptor and that there is a family of mammalian ARF proteins, of which we have cloned and expressed six. Each of them enhanced cholera toxin ADP-ribosyltransferase activity in a GTP-dependent fashion, although their requirements for activity were distinctly different. This is probably reflected in the differences noted earlier in requirements for stability and activity of ARF purified from different tissues.

While searching for previously unrecognized members of the ARF family, a clone was isolated that encoded an ARF-like domain of 172 amino acids. Further cloning yielded more upstream sequence resulting in identification of a putative 64-kDa protein that contains a ~18 kDa ARF domain at its carboxyl terminus. This was named ARD 1 (for ARF domain). Human and rat ARD 1 were remarkably similar, with coding region nucleotide and deduced amino acid sequences 92 and 98% identical, respectively. The nucleotide sequence of the ARF domain of human ARD 1 is 60-66% identical to those of mammalian ARFs. Of the four amino acid sequences believed to be involved in guanine nucleotide binding and GTP hydrolysis, GLDGAGK, DVGG, and NKQD are identical in ARD 1 and the ARFs. Only CAT, which is found in ARFs, differs in ARD 1 where it is DAR, with the apparently critical alanine conserved.

Although its physiological function is not yet defined, ARD 1 has been of interest in regard to ARF structure and function. Its ARF domain lacks the 15 N-terminal amino acids of mammalian ARF sequences that had been postulated to be critical for ARF activity. ARD 1, nevertheless, activated cholera toxin. The ARF domain expressed by itself as a recombinant protein was much less active. The activity of a fusion protein of glutathione-S-transferase and the ARF domain was significantly greater, consistent with the notion that although toxin activation does not require the amino terminal 15 amino acids, that portion of the molecule may be important for overall protein conformation or stability or interaction with lipids. More than a decade ago, other workers described a protein distinctly larger than ARF that possessed ARF activity. We speculate that ARD 1 is related to that protein and represents a new family of larger GTP-binding proteins that contain a domain with ARF structure and another domain of unknown function. The ubiquitous distribution of two ARD 1 messenger RNAs provides no clues, but perhaps, like ARFs, they have a role in intracellular membrane trafficking.

The mammalian ARFs fall into three classes based on deduced amino acid sequence, size, phylogenetic analysis, and gene structure. Class I ARFs (ARFs 1, 2, and 3) are ~96% identical in amino acid sequence and sites of intron-exon junctions in their genes are identical. It seems likely that functional specificity is achieved, at least in part, by regulation of expression of individual ARF genes in different cells or at different times during

development. We had found some time ago that amounts of ARF 3 (relative to ARF 1) increased in rat brain between the second and the 27th post-natal day. Similarly, ARF 3 mRNA increased over this period whereas amounts of mRNAs for other ARFs either declined or remained constant. To identify cis-acting elements responsible for expression of the human ARF 3 gene, fusion genes with different amounts of 5'-flanking region of the ARF 3 gene linked to a bacterial CAT or luciferase reporter gene were constructed. For these constructs, a procedure for ligation-independent cloning that we devised last year was used. Reporter plasmids were modified to facilitate preparation of defined promoter deletions. The chimeric genes were transfected into cultured human IMR-32 neuroblastoma cells, which express an endogenous ARF 3 gene.

The 5'-flanking region of the human ARF 3 gene has features resembling those of a "housekeeping" gene. It lacks a TATA or CAAT box, has several GC-boxes within a highly GC-rich region, and uses multiple transcription initiation sites. Sequences within 58 bp of the transcription initiation site were necessary for full expression, particularly a sequence containing the 10-bp palindrome TCTCGCGAGA. Nuclear extracts from IMR 32 cells contained a protein that bound to an oligonucleotide containing this palindrome. Mutation within the palindrome abolished in vitro binding of the protein, which was termed TLTF for TATA-Less transcription factor. This DNA-binding protein apparently also interacts with a sequence upstream of the palindrome, consistent with the possibility that the same factor binds to two promoter elements. Effects of a mutated palindrome were consistent with the conclusion that this region functions physiologically to control expression of the ARF 3 gene.

ARFs apparently have physiological roles in intracellular membrane trafficking processes such as endocytosis and nuclear membrane assembly, in addition to vesicular transport of proteins from the endoplasmic reticulum to Golgi compartments. It seemed reasonable that the several mammalian ARFs should have different functions and likely, different intracellular localizations. Consistent with this hypothesis, we had found last year that ARFs 1, 3, and 5 clearly differed in their binding to Golgi and other subcellular membrane fractions. Since association of ARF with Golgi involves other soluble proteins and since Golgi membranes are disrupted in a characteristic fashion by the fungal metabolite Brefeldin A (BFA) we investigated whether BFA and/or soluble accessory proteins ($M_r > 40k = SAP$) might affect differently the association of ARFs 1, 3, and 5 with Golgi membranes. Individual ARFs 1, 3, and 5 were identified and their amounts estimated after electrophoretic separation by reaction with antibodies specific for class I or class II ARFs. Findings and conclusions were similar whether single purified ARFs (ARFs 1 and 3 from bovine brain cytosol, recombinant myristoylated ARF 5 synthesized in *E. coli*) or a partially purified fraction of mixed ARFs from rat brain were used.

ARF binding to membranes (or to certain phospholipids, as we earlier reported) requires that it be bound to GTP or an analogue. In the presence of GTP γ S, a non-hydrolyzable GTP analogue, SAP increased the binding of ARFs 1 and 3, but not ARF 5, to a Golgi membrane fraction. BFA inhibited only the fraction of ARF binding that was dependent on SAP; i.e., it did not inhibit binding of ARF 5 and inhibited only the increment in ARF 1 or ARF 3 binding induced by SAP. We believe that some, perhaps large, fraction of ARF binding observed in experiments such as ours is "non-specific", analogous to the GTP-dependent binding of ARF to phospholipids. The BFA inhibition of binding of ARFs 1 and 3 would appear to define one relatively specific component, i.e.,

that which is dependent on SAP. The active component in SAP is probably a protein that promotes guanine nucleotide exchange (or stimulates GDP release), specifically for ARFs 1 and 3. Presumably another protein or proteins plays a similar role for ARF 5 and other ARFs. Alternatively, ARF5 can interact functionally with the exchange protein but does not bind specifically to any of the membranes in the Golgi preparation used. Purification of the guanine nucleotide exchange protein is in progress after establishment of two assays to measure more directly GTP or GTP γ S binding by a specific ARF protein.

The substrate for the A subunit of cholera toxin in disease is a specific arginine in the α -subunit of G_{ss} , the stimulatory G protein of the adenylyl cyclase system that may also regulate certain ion fluxes. The toxin also catalyzes the ADP-ribosylation of simple guanidino compounds (e.g., free arginine, agmatine), and several other substrates. The assumption was made that recognition of G_{ss} would require the participation of more of toxin structure than would ADP-ribosylation of a simple guanidino compound like agmatine. Based on structural analogies between cholera toxin and other bacterial toxins such as pertussis toxin, several mutant CTA 1 molecules were synthesized in *E. coli* and tested for their ability to ADP-ribosylate agmatine, G_{ss} , and transducin (G_{ta}), as well as to auto-ADP-ribosylate. Several mutations resulted in overall loss of enzymatic activity. Replacement of histidine-70 with asparagine, however, diminished ADP-ribosyl- G_{ss} formation more than it did ADP-ribosyl-agmatine synthesis, consistent with the view that histidine-70 may be involved in the specific recognition of G_{ss} by cholera toxin.

Identification of the epitopes of two monoclonal antibodies against the α -subunit of transducin (G_{ta}), the GTP-binding protein of the retinal photoreceptor system has continued this year. By all physical mapping studies, including cleavage with specific proteases and expression of the recombinant protein in *E. coli* with and without myristoyl modification of the amino terminal glycine, they appear to be identical. Functionally, however, the antibodies differ in that only one of them inhibits the transducin $\beta\gamma$ -dependent ADP-ribosylation of G_{ta} . Evidence of several types has implicated the amino terminus of G_{ta} in its interaction with the $\beta\gamma$ subunits, which is why this functional assay was used after the proteolysis experiments had mapped the antibody epitopes to include the terminal ~ 15 amino acids plus the myristoyl moiety.

ADP-ribosylation of Proteins in Animal Cells

Mono-ADP-ribosylation is a post-translational modification of proteins, involved in the action of bacterial toxins (e.g., pertussis toxin, cholera toxin) on mammalian cells. In a putative ADP-ribosylation cycle, an ADP-ribosyltransferase catalyzes the forward reaction, ADP-ribosylation of arginine residues in proteins, and ADP-ribosylarginine hydrolases catalyze the opposing reaction, cleavage of the ADP-ribose-arginine bond, releasing ADP-ribose and regenerating free arginine. Hydrolases have been identified in many mammalian and avian tissues and species and cloned from rat brain. NAD:arginine ADP-ribosyltransferases were identified as well in many eukaryotic cells and tissues. The presence in animal cells of NAD:arginine ADP-ribosyltransferases was first demonstrated in this laboratory. Attempts to isolate a cDNA clone for a mammalian transferase had, however, been unsuccessful until this year. A transferase was purified from rabbit skeletal muscle that yielded amino acid sequence from seven tryptic peptides. Based on this information, degenerate oligonucleotides were prepared and used for PCR

amplification of a rabbit skeletal muscle cDNA library. Finally, a composite cDNA sequence was obtained. It contained a 981-base-pair open reading frame that encoded a 36,134-kDa protein. The deduced amino acid sequence contained sequences of all seven tryptic peptides. There were two potential sites of N-linked glycosylation. The hydrophilicity plot showed hydrophobic N- and C-termini with a hydrophilic center, i.e., an overall structure characteristic of a glycosylphosphatidylinositol-anchored membrane protein. A truncated form of the protein (amino acids 24 to 303), lacking the hydrophobic N- and C-termini, synthesized in *E. coli* exhibited ADP-ribosyltransferase activity. A specific oligonucleotide probe hybridized with a 4-kb mRNA found primarily in skeletal and cardiac muscle. Work continues to identify the substrate (or substrates) for this enzyme as well as to determine the functional significance of the putative glycosylphosphatidylinositol modification.

While looking for a cysteine-specific ADP-ribosyltransferase in animal cells, we had found last year 1) the nonenzymatic reaction of ADP-ribose (generated from NAD by ubiquitous NAD glycohydrolase activity) with free cysteine to yield a product identified as ADP-ribose thiazolidine carboxylic acid and 2) the nonenzymatic reaction of ADP-ribose with a catalytic cysteine in the active site of aldehyde dehydrogenase with resulting inactivation. During that time there were several reports implicating nitric oxide (NO) in the regulation of endogenous ADP-ribosylation of cysteines in proteins. The NO-stimulated, NAD-dependent modification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was investigated in a defined system with purified enzyme. Although GAPDH was inhibited >60%, <2% of the enzyme was covalently modified with NAD. In addition, the modification was clearly not ADP-ribosylation, but included both the adenine and nicotinamide portions of the NAD molecule. Both of these moieties were likewise part of the product released from the modified enzyme by chemical treatment, but the released compound differed from NAD. Its structure is still being investigated. The covalent modification of GAPDH with NAD clearly did not explain the inhibition, which is probably due to modification of the active site cysteine by NO itself.

Of nine other enzymes tested for NAD modification stimulated by NO, only alcohol dehydrogenase was modified, and to <10% of the extent of GAPDH. Aldehyde dehydrogenase was inhibited by NO to the same extent as GAPDH but was not modified at all with NAD, further supporting the conclusion that S-nitrosylation in itself is sufficient for inhibition of certain dehydrogenases. Bovine serum albumin (BSA) was reported to be modified similarly with NAD in the presence of NO, and so might have provided a model reaction for characterization of the modification in a large-scale preparation. Investigation of the modification of BSA showed that like the modification of GAPDH, it involved the entire NAD molecule. In contrast to that of GAPDH, however, the modification of BSA was not sensitive to Hg^{2+} treatment, indicating that a different type of chemical linkage was involved.

Prior to this work, the prevailing view of the NO-stimulated NAD-dependent modification of GAPDH was that it represented auto-ADP-ribosylation, suggesting that NO stimulates a latent auto-ADP-ribosyltransferase activity of the enzyme. Our results indicate that the modification of GAPDH could be considered the result of an unexpected chemical reactivity of a nitrosothiol group on the enzyme, so that perhaps the nitrosothiol (or its components) forms a covalent bond with NAD. Alternatively a thiol-NAD-NO linkage may be formed.

Annual Report of the Laboratory of Chemical Pharmacology
October 1, 1992 to September 30, 1993

In the past this Laboratory has focused on studies related to drug-induced hypersensitivity reactions. For example, the Section on Pharmacological Chemistry studies the mechanisms by which chemical reactive metabolites, such as the trifluoroacetyl halide formed from halothane, are synthesized and form neoantigens, and the Section on Cellular Pharmacology studies the mechanisms by which antigens cause the cascade of events that lead to the release of inflammatory mediators from mast cells. During the past two years, however, the Laboratory has expanded its interests into several other areas. The Section of Enzyme Drug Interaction studies factors that govern the substrate specificity and product formation by the various cytochrome P-450 enzymes. The Section on Drug Tissue Interaction studies the expression of messenger RNA for tissue specific isoforms of various enzymes, such as guanylate cyclase and heat shock proteins. A small group studies mechanisms of dopamine uptake and release. In addition, the Sections have collaborated on studies of (a) mechanisms of drug induced alterations of hemoproteins including cytochrome P-450 enzymes and (b) the biochemistry and pharmacology of nitric oxide.

Mechanisms for Activation of Mast Cells

It is now believed that mast cells and blood basophils, when stimulated with antigens, mediate immediate hypersensitivity reactions through rapid degranulation and discharge of inflammatory mediators. They also mediate delayed inflammatory responses through increased expression of a repertoire of cytokines. These reactions result in symptoms commonly associated with seasonal and drug-induced allergies (e.g. urticaria and sneezing) and the chronic inflammatory conditions that ensue from some of these allergies (e.g. airway hypersensitivity in asthma). Our objective is to delineate the signalling pathways for each of the responses described above. The model for our studies is the cultured rat RBL-2H3 cell, which like mast cells and basophils contain receptors (of the FcεR1 category) for immunoglobulin E (which bind antigen), and RBL-2H3 cells that have been transfected with the gene for the muscarinic m1 receptor. The cells also contain adenosine receptors which we had previously characterized as being distinct from the classic adenylyl cyclase coupled adenosine A₁ and A₂ receptors (J. Biol. Chem. 265:745, 1990). This year we have identified the receptor as the recently cloned adenosine A₃ receptor and shown that it is present in other types of mast cells as well as T cells and endothelial cells (J. Biol. Chem. in press). Also the finding that mast cells contain neither of the classic adenosine receptors clarifies paradoxical findings reported in the literature, for example, the inefficacy of methylxanthines in blocking the actions of adenosine and the insignificant effects of adenosine on cyclic AMP levels in tissue mast cells.

The presence of intrinsic (i.e. Fcε and A₃) and transfected (i.e. m1) receptors in RBL-2H3 cells has allowed assessment of the importance of various transducing components in the signalling pathways in RBL-2H3 cells. Work this year and the accumulating evidence over several years have demonstrated the following. All three types of receptors utilize distinct coupling mechanisms for mediation of signals across

the plasma membrane, the A_3 and $m1$ receptors via G proteins (identified as G_q for the $m1$ receptor and tentatively as G_{i-3} for the A_3 receptor) and, as demonstrated by others and confirmed by us, the $Fc\epsilon$ receptor via recruitment of a cytosolic tyrosine kinase and tyrosine phosphorylation of various proteins including the $\gamma 1$ isozyme of phospholipase C. However all three types of receptors allow activation of the phospholipases A_2 , C and D, mobilization of Ca^{2+} from the same intracellular pool and replenishment of this pool by influx of external Ca^{2+} through the same influx mechanism. These events culminate in the activation of protein kinase C (PKC) and Ca^{2+} -dependent myosin light chain kinase with the phosphorylation of myosin light chains by both enzymes. A notable distinction, however, is that adenosine analogs cause only transient activation of phospholipase C, and as a consequence transient mobilization of Ca^{2+} and Ca^{2+} -dependent phosphorylation, but sustained activation of phospholipase D, and as a consequence sustained activation of PKC and phosphorylation by PKC. The net effect is that the cells do not release inflammatory mediators but signals for such release are markedly synergized when cells are subsequently stimulated via $Fc\epsilon$ or muscarinic $m1$ receptors with low concentrations of antigen and carbachol (a muscarinic agonist). This work clearly demonstrated that phospholipase D provides a potent synergistic stimulatory signal in RBL-2H3 cells. We also discovered that whereas antigen and carbachol are equally effective in stimulating the rapid discharge of inflammatory mediators, carbachol is a weak stimulant for production of the cytokine TNF_α compared to antigen; a possible indication that the recruitment of tyrosine kinase by antigen is essential for activation of the gene for TNF_α .

We have now identified (by two dimensional chromatography and antibody mapping) an additional transducing element that acts at a late step in the stimulatory pathway, namely the Mitogen Activating Protein kinase, MAP^{p42} , which is thought to be a co-ordination element for regulating cell division. Stimulation of cells via all three receptors as well as by activators of PKC and Ca^{2+} -mobilizing agents caused formation of multiple tyrosine phosphorylated forms of MAP^{p42} . Without exception, the protein was not activated when secretion was blocked by use of various inhibitors of early signals. This finding is significant because activation of the kinase is dependent on mobilization of both Ca^{2+} and PKC, which, as demonstrated last year, are necessary intermediate events for secretion. Finally, cloning of the gene for the G protein (p100) is now well advanced and has revealed several novel features for this protein.

Mechanisms of Drug-Induced Toxicities

Neoantigens of halothane: Halothane ($CF_3CHClBr$) causes a severe and frequently lethal hepatic necrosis, presumably by a hypersensitivity reaction. Patients suffering from this halothane-induced liver necrosis produce antibodies that react with several hepatic proteins in animals treated with halothane. Since these findings raised the possibility that the necrosis might be caused by reactions of one or more of the antibodies with altered proteins on the surface of hepatocytes, the Section on Pharmacological Chemistry embarked on a program to identify the neoantigens. During the past few years they have identified all of the major ones. However, the function

of a 58 kDa protein remained obscure. Immunohistochemical studies with antibodies formed against the protein revealed that it is preferentially located in specific cells of several organs; for example the ganglionic cells of the heart. In a further attempt to identify its function, the Section has attempted to obtain clones of its cDNA from a human library. Several clones have been isolated and their CDNAS are being sequenced.

Presumably, neoantigens must be processed before they induce the formation of antibodies. During the past year, the Section has demonstrated that trifluoroacetylated neoantigens are processed in parenchymal cells rather than in Kupffer or endothelial cells; little if any of the neoantigens are released intact from the parenchymal cells. The enzyme that catalyzes the processing appears to be a cellular serine-protease.

Nonsteroidal anti-inflammatory agents: Several nonsteroidal antiinflammatory agents cause idiosyncratic hepatitis, hemolytic anemia, agranulocytosis and kidney damage. Two years ago the Section on Pharmacological Chemistry, using an antibody against diclofenac, one of these drugs, discovered that a metabolite of the drug became covalently bound to a 110 kDa protein in liver. This past year, the Section has shown that the target protein is localized in the plasma membrane of hepatocytes and the covalent binding is mediated by the 1-O-acyl glucuronide of the drug.

Mechanisms of Metabolism-based Alterations of Hemoproteins

Although many chemically reactive metabolites are sufficiently long lived to escape the enzymes that catalyze their formation and to become covalently bound to many other proteins and other macromolecules, some chemically reactive metabolites are short lived and never leave the active site of the enzyme. These short lived metabolites may inactivate hemoproteins by 1) reacting with the heme, 2) reacting with amino acid groups in the active site of the heme enzyme, or 3) reacting with either the protein or the heme to form radicals that lead to the covalent binding of the heme to the protein. Occasionally, a "long-lived chemically reactive metabolite" may also inactivate an enzyme by entering the active site of the enzyme for which it has high affinity and reacting with groups within it. During the past few years, the laboratory has discovered systems illustrating each of these mechanisms of inactivation.

Myoglobin: In the past Dr. Osawa has shown that bromotrichloromethane (BrCCl_3) and hydrogen peroxide cause the covalent binding of the heme to protein. Moreover, the alteration shifted the myoglobin from an oxygen storage protein to an oxidase, which during redox cycling forms superoxide and hydrogen peroxide and leads to the further destruction of the myoglobin. The formation of superoxide and hydrogen peroxide by the altered myoglobin thus raised the possibility that the alteration could lead to cellular death. In accord with this view, Osawa and his collaborators have shown that incorporation of altered myoglobin into fiberblasts led to cell death, whereas incorporation of native myoglobin did not.

Prostaglandin H synthase: Hydrogen peroxide causes the covalent binding of the heme to the apoprotein of prostaglandin H synthase. Partial proteolysis followed by HPLC separation of the peptides and mass spectrometric analysis indicated that the

heme appears to be attached to tyrosine 385. If this is confirmed, it would be the first direct evidence that tyrosine 385 is in the active site of the enzyme.

Neutrophil oxidase: Arachidonic acid inactivates this multi-protein complex with the concomitant alteration of the heme prosthetic group. The addition of a cytosolic protein (p47) protects the complex from inactivation and prevents the alteration of the heme.

Horseradish peroxidase: The porphyrinogenic agent, 3,5-dicarboxy-3,6-dimethyl-4-ethyl-1,4-dihydropyridine (DDEP), destroys various cytochrome P-450 isozymes by different mechanisms. Inhibition of cytochromes P-450 2C11 and 2C6 appear to be inhibited through ethylation of the heme prosthetic groups, whereas inhibition of cytochrome P-450 3A occurs through covalent binding of the heme to the apoprotein. Consequently, addition of heme can reactivate 2C11 and 2C6, but not 3A. Since adequate amounts of these isoforms of cytochrome P-450 are not available, Dr. Sugiyama has studied the oxidation of DDEP by $K_3Fe(CN)_6$ and by a combination of horseradish peroxidase and H_2O_2 . Both systems form 3,5-dicarboxy-3,6-dimethyl-4-ethyl-pyridine (EDP) and 3,5-dicarboxy-3,6-dimethyl-pyridine (DP), but the relative amounts depended on the pH of the medium; under neutral and acetic conditions EDP predominated, whereas under basic conditions DP predominated. Moreover, under basic but not acidic conditions, horseradish peroxidase was inactivated and the heme group was altered. A combination of mass spectroscopic analysis and NMR indicated that the altered heme contained a 2-hydroxyethyl group attached to the δ -meso carbon of the heme. Similar experiments were performed with ethylhydrazine as the potential ethyl group donor.

From these results and from calculations of the thermodynamics of various reaction pathways, the following mechanism of DDEP metabolism catalyzed by cytochrome P-450 isozymes was postulated. First, DDEP undergoes a one-electron oxidation by all sensitive P-450s, but the fate of the radical cation differs with the isoform. The predominant formation of DP by P-450 2C11 and 2C6 indicates that the active sites of these isozymes are either basic or very lipophilic and loose, which facilitates the deprotonation of N-hydrogen of a N radical cation and the subsequent formation of DP with the release of an ethyl radical that reacts with the heme. On the other hand, P-450 3A forms predominantly EDP, which suggests either that the active site is more open to the solvent or is acidic. This could explain why P-450 3A is not inactivated by formation of the ethyl radical altered heme. The mechanism for inactivation by covalent binding of the heme to the apoprotein, however, still remains obscure.

Synthesis and Actions of Nitric Oxide

Dopamine release: Last year Dr. Hanbauer reported that, in slices of striatum from rat brain, nitric oxide mediates the release of dopamine from presynaptic neurons evoked by N-methyl-D-aspartate, a postsynaptic glutamate receptor agonist. Since it is known that nitric oxide activates guanylate cyclase, it seemed plausible that the mechanism for dopamine release might be mediated by cGMP, but studies during the past year make this untenable. Carbon monoxide, which also activates guanylate cyclase, and 8-bromo-cGMP, which increases cGMP in cells, do not cause the release

of dopamine. Moreover, nitric oxide does not increase unbound intracellular calcium in dopaminergic cells, but does increase dopamine release in the slices in the absence of external calcium. The mechanism of release thus remains to be clarified.

Cytochrome P-450 enzymes: Last year Dr. Osawa reported that nitric oxide inhibits cytochrome P-450 enzymes both reversibly and irreversibly. Since it is known that various disease states lead to decreases in cytochrome P-450 in liver, it seemed possible that part of the decreases might be due to the formation of nitric oxide. In accord with this view, Dr. Darbyshire found that the induction of nitric oxide synthesis by cytokines in primary cultures of rat hepatocytes lead to the decrease in the metabolism of testosterone by cytochrome P-450 enzymes. The decrease could be partially prevented by the addition of N-methylarginine, an inhibitor of nitric oxide synthesis. Since the cytokines also change the pattern of metabolites of testosterone as well as the total rate of metabolism, it seems likely that some of the isoforms of cytochrome P-450 are more sensitive to the impairing effects of nitric oxide than are others.

Platelet enzymes: Metabolism of arachidonic acid by lipoxygenase and prostaglandin H synthase in human platelets is decreased by incubation with L- but not D-arginine, suggesting that nitric oxide inhibits these enzymes. Sodium nitroprusside, which is a precursor of nitric oxide and inhibits platelet aggregation, also inhibits arachidonic acid metabolism.

Brain nitric oxide synthase: Phencyclidine, a psychotomimetic drug of abuse, irreversibly inactivates the enzyme.

Mechanisms of Cytochrome P-450 Enzymes

Unlike most enzymes, many cytochrome P-450 enzymes form several metabolites from the same substrate. Several may be envisioned by which the various metabolites might be formed. In each of the mechanisms, the substrate combines with the enzyme in different orientations to form complexes that then are activated to a set of (EOS) complexes. The fate of these (EOS) complexes determines the mechanism. In the parallel pathway mechanism, the (EOS) complexes are so stable and rigid they cannot be converted either directly or indirectly to complexes with different orientations; the orientation of the (ES) complex thus determines which metabolite will be formed. In the nondissociative mechanisms, the complexes are not rigid; instead they undergo interconversion while the substrate remains in the active site of the enzyme. In the dissociative mechanisms, the (EOS) complexes dissociate to (EO) and (S) but recombine to form (EOS) complexes with either the same or different orientations.

We have derived steady-state rate equations for each of these mechanisms and used them to elucidate the mechanism by which P-450 2C11 converts d_0 -testosterone and d_5 -testosterone to 2 α -hydroxytestosterone on the one hand and 16 α -hydroxytestosterone and androstenedione on the other. The results reveal that the metabolites are not formed by the nondissociative mechanisms. Some of the results indicate that the metabolites are formed by the general dissociative mechanism, but other results indicate that the parallel pathway mechanism may be invoked under certain conditions. When the system acts by the parallel pathway

mechanism, the finding of a normal isotope effect on V_{max}/K_m for the total metabolism of testosterone would indicate that the activated enzyme substrate complex, (EOS), must be reduced to water. By contrast, when the dissociative mechanism predicts a normal isotope effect even when reduction to water does not occur.

Other Studies

Expression of mRNA of enzymes and proteins in specific tissues: There frequently are many different forms of enzymes produced in the body, but the expression of these different forms frequently depends on the tissue. Although the different forms sometimes may be identified by specific immunohistological techniques, such techniques are not always feasible.

Guanylate cyclase: During the past few years Dr. Krishna has been developing ways of detecting the expression of several enzymes and other proteins by reverse transcription of MRNAS from human retina and amplifying the resulting CDNAS by the polymerase chain reaction (PCR). During the past year he has obtained a PCR product for guanylate cyclase-A from human retina mRNA after reverse transcription to cDNA and used it as a hybridization probe to screen a human retinal library (λ gt11). Two clones were isolated, one of which was a false positive. He plans to sequence the other clone.

Heat shock factor: Exposure to heat and other stimuli frequently results in the increased expression of several "heat shock proteins", such as heme oxygenase. The expression appears to be governed by "heat shock factors" (HSF), which bind to "heat shock elements" (HSE) in the gene. Since Dr. Krishna's Section has shown that heme oxygenase is induced in the retina, he has embarked on a program to determine whether the human retina expresses the mRNA for HSF and if so to identify it. During the past year he has isolated several cDNA clones of HSF from a human retinal library and plans to sequence each of them.

Calmodulin binding domains: Polypeptides representing the four binding domains of calmodulin have been synthesized and used to study binding characteristics by NMR. Dr. Krishna has found that in the absence of calcium, the polypeptide representing the fourth calcium binding domain (which is the high affinity binding site) adopts a random structure, but in the presence of calcium it becomes rigid. Moreover, the 16 amino acids preceding the calcium binding region shift from a random coil structure to an alpha helical structure on addition of calcium.

Neurotoxicity in dopamine-containing neurons: Dr Hanbauer has found that high concentrations ($50 \mu M$) of glutamate receptor agonists, such as glutamate, kainate, quisqualate, or AMPA, cause an increase in $[Ca^{2+}]_i$, which is irreversible in dopaminergic neurons but not in non-dopaminergic neurons. These treatments of the dopaminergic neurons result in cell death several hours later, but cell death can be prevented by treatment with dantrolene, a blocker of Ca^{2+} release from intracellular stores.

**Annual Report of the Clinical Hematology Branch
National Heart, Lung and Blood Institute
October 1, 1992 to September 30, 1993**

The research of this Branch is directed toward understanding the underlying causes and developing effective treatment for major hematological disorders, including thalassemia, sickle cell anemia, and various syndromes of bone marrow failure and myelodysplasia. The scope of our work is broad and includes basic study of the molecular mechanisms of gene regulation and extends to applied clinical trials of specific therapeutic agents. Modern methods of molecular and cell biology, including recombinant DNA technology, are utilized in the comprehensive approach to disease mechanisms and therapy.

Several organizational changes have been initiated during the past year and will be completed during the next several months. The Cell Biology Section under Dr. Neal S. Young has been converted to a new branch, the Hematology Branch, for which a separate annual report has been submitted. With the departure of its Branch Chief, Dr. Arthur W. Nienhuis, the Clinical Hematology Branch will close during the next year. Several projects focusing on development of gene transfer into hematopoietic stem cells as a therapeutic tool will continue in the newly formed Hematology Branch. Efforts to define the molecular basis for hypertrophic cardiomyopathy will continue in collaboration with investigators in the Cardiology Branch.

The following are several highlights of progress made in our research during the past year.

Gene Insertion into Hematopoietic Stem Cells

Gene replacement as therapy for genetic and acquired bone marrow disorders has become a realistic research goal. Our efforts have focused on the biology of hematopoietic stem cells and means to manipulate these cells in vitro to achieve gene insertion. Most stem cells are quiescent and in the G₀ phase of the cell cycle. Retroviral mediated gene transfer requires cell division. We have established the ability of cytokine combinations to enhance gene transfer into murine stem cells in vitro. Administration of G-CSF and stem cell factor, two cytokines that act on early hematopoietic cells, to splenectomized mice results in a massive mobilization of repopulating stem cells. Peripheral blood from these animals provide a convenient, readily accessible source of cells for gene transfer. Using a combination of centrifugal elutriation and positive and negative immunoselection, we have purified murine stem cells more than a 1,000 fold. Reconstitution of individual animals can be achieved with as few as 100 cells. The biochemical and physiological properties of these highly purified stem cells are being defined with the goal of finding mechanisms to render such cells more

susceptible to gene transfer.

Insights acquired from studies in the murine model are applied in an autologous bone marrow transplantation model in non-human primates. Early hematopoietic stem cells are purified from rhesus bone marrow by positive immunoselection, cultured in vitro with recombinant growth factors in the presence of retroviral vectors and transplanted after the animal has received total body irradiation to ablate remaining bone marrow cells. Two animals that received autologous stem cells transduced with a vector containing the gene for adenosine deaminase (ADA) have been followed for more than two years. The proviral genome has been shown to be present in cells of the myeloid, T-lymphoid and B-lymphoid lineages suggesting that a multipotential repopulating stem cell has been the target for gene transfer. Expression of the ADA gene in T-lymphocytes has been documented establishing the potential efficacy of stem cell targeted gene insertion for treatment of severe combined immunodeficiency due to a defect in the ADA gene. Using positive immunoselection for cells co-expressing the CD34 and Thy1 antigens, we have achieved reconstitution of irradiated recipients with as few as 5×10^5 cells per kilogram. These highly purified stem cell populations can now be manipulated in vitro in an effort to find conditions that permit gene transfer.

We have begun a program of autologous bone marrow transplantation for patients with multiple myeloma and chronic myelogenous leukemia. Similar studies in patients with breast cancer are being conducted in collaboration with investigators in the Medicine Branch of the National Cancer Institute. Six patients have received peripheral blood and bone marrow cells transduced with retroviral vector preparations. Genetically modified cells have been detected in the peripheral blood during the early stages post-transplantation. Vectors have been developed that are suitable for transfer of the human multi-drug resistance gene into primitive hematopoietic cells. During the next year, we anticipate initiating a protocol designed to determine whether cells containing and expressing the MDR1 gene can be selected in vivo in patients who receive chemotherapy after recovery from bone marrow transplantation. The ultimate goal of these studies is to use gene transfer to create bone marrow resistant to myelosuppression by commonly used chemotherapeutic agents. We have also used retroviral mediated gene transfer to correct the genetic defect in hematopoietic cells of patients with Fanconi's anemia. This congenital hypoplastic anemia reflects a defect in DNA repair. One gene that may be defective in such patients has been molecularly cloned and used in developing vectors that have the potential for being useful in the genetic therapy of this disorder.

Regulation of Hemoglobin Switching

Patients with either severe β -thalassemia or sickle cell anemia could benefit from increased production of fetal hemoglobin. At the gene level, the switch reflexes turn-off of the γ -globin genes and turn-on of the β -globin genes. If both β genes are defective, the switch leads to the onset of hematological disease. The β

and γ genes are part of a multi-gene cluster on chromosome 11 that extends over 60,000 base pairs of DNA. Human globin genes exhibit tissue and developmental stage specificity. Gene expression increases dramatically during progression from pro-erythroblasts to the latter stages of erythropoiesis. Regulation of the globin genes is achieved by factors that bind DNA with sequence specificity (trans-acting factors). These proteins bind to cis-acting elements within and flanking the individual genes in modulating gene expression. In addition, several regulatory elements are distributed in the 20 kilobases of DNA upstream from the cluster; these elements work coordinately in establishing high level globin gene expression in erythroid cells. This complex of regulatory elements is referred to as the locus control region (LCR).

Studies in prior years have established that various pharmacological agents are able to augment HbF synthesis in patients with sickle cell anemia and thalassemia. However, the real solution to therapeutic hemoglobin switching will come from a thorough knowledge of the regulatory mechanisms that control gene expression. Accordingly, most of our efforts have focused on identifying specific cis-active elements involved in switching. These include a powerful enhancer within the locus control region upstream from the cluster, a stage selector element within the γ -globin gene promoter and a regulatory element downstream from the γ -globin gene. Specific proteins that interact with each of these elements have been identified by a combination of functional and DNA binding assays. Our work has focused on purifying these proteins and obtaining molecular clones of their coding sequences.

Nuclear factor-erythroid 2 (NF-E2) is responsible for the inducible activity of the powerful enhancer within the LCR. We have purified both components of human NF-E2 and, in collaboration with N. Andrews and S. Orkin of Children's Hospital in Boston, obtained molecular clones of their coding sequences. The proteins, p45 and p18, are basic leucine zipper proteins that form a heterodimeric complex having the activity and DNA binding specificity of NF-E2. Another protein that binds to sequences within the γ promoter and a downstream regulatory element has been biochemically purified and sequences of several of its protease peptides determined. This protein is identical to SATB-1, a previously characterized protein that binds to DNA sequences that interact with the nuclear matrix. These observations suggest that the downstream regulatory element may form a boundary in chromatin and/or interact with the γ promoter by mutual binding of these regulatory elements to the nuclear matrix.

Earlier we had identified a stage selector element in the γ promoter that, through its binding to a specific protein, facilitates interaction with the powerful enhancer in the locus control region. The protein, termed stage selector protein (SSP) has now been purified. SSP binds to the stage selector element competitively with another ubiquitously expressed transcriptional activator, Sp1. Methylation of CpG dinucleotides within the stage selector element enhances binding of Sp1 at the expense of SSP. Methylation of these nucleotides is

characteristically present in adult erythroid cells in which the γ gene is silenced. These observations support an important role for SSP in modulating hemoglobin switching.

Molecular Defect in Hypertrophic Cardiomyopathy

Hypertrophic cardiomyopathy is a dominantly inherited genetic disease of the heart, manifested by the left ventricular outflow obstruction, cardiac failure, arrhythmia and/or sudden death. Investigators in the Clinical Hematology Branch in collaboration with researchers in several other branches at the NIH have undertaken a genetic, molecular biological and functional approach to this disorder. The cardiac myosin heavy chain gene is the disease gene in 10-30% of affected kindreds. Thirteen distinct mutations in the head or head-rod junction region of the myosin molecule have been identified in 17 different kindreds. Pre-symptomatic diagnosis can now be accomplished in these families. Mutation-specific natural histories are being defined; certain mutations are associated with high penetrance, early onset of disease and a high frequency of sudden death, whereas, other mutations have a much more benign clinical course. the cardiac myosin heavy chain gene is also expressed in skeletal muscle. Our studies have shown defects in myosin contractility *in vitro*, diminished isometric force generation by single myofibers and characteristic histopathological abnormalities in skeletal muscle of affected patients.

HEMATOLOGY BRANCH

The major research and clinical interests of the Hematology Branch are in normal hematopoiesis and especially in the pathogenesis, pathophysiology, and effective treatment of bone marrow failure states. Administratively, the Hematology Branch was created in January of 1993 and incorporated the Cell Biology Section and other elements of the Clinical Hematology Branch. The Branch consists of research groups in the following areas: B19 parvovirus; bone marrow failure; including viral and immune pathophysiology; gene therapy; and bone marrow transplant. The Hematology Clinical Service has been relocated from its former quarters on 8 East to newly renovated space on 2 West. A major addition has been the recruitment and hiring of Dr. John Barrett to head a newly formed Bone Marrow Transplant Unit. The spectrum of clinical diseases treated on this service has expanded to include not only acquired aplastic anemia, single lineage bone marrow failure syndromes, Fanconi's anemia, and congenital hemoglobinopathies, but also multiple myeloma and chronic myelogenous leukemia, the latter two treated by autologous bone marrow transplantation. The Hematology Branch also administers an accredited and successful Hematology Fellowship Program.

B19 Parvovirus

Advances have been made in three areas: 1) structural studies, including identification of the cellular receptor; 2) immunogenicity, including vaccine development; 3) purification and partial characterization of the viral non-structural protein.

The Cellular Receptor and B19 Structure

A suitable explanation for the extraordinary erythroid tropism of B19 parvovirus has been found by identification of its cellular receptor. B19 parvovirus infects and replicates only in erythroid progenitor cells. We have previously identified hemagglutination as a property of the virus, hemagglutination correlated with infectivity. Inhibition of hemagglutination was achieved by red cell extracts of the lipid rather than the protein portion of membranes. The active inhibitory principle had the properties of a neutral glycolipid, and when purified neutral glycolipids were examined in the assay, globoside, or tetrahexose ceramide, was extremely potent. Globoside is P antigen, a red cell antigen. The biological significance of P antigen was determined in erythroid colony assays. Excess of soluble globoside competed for virus binding sites and blocked infectivity. Monoclonal antibody to P antigen also blocked infectivity. These results identified P antigen as the parvovirus receptor. Rare normal individuals genetically lack P antigen (p or Tja- phenotype). We tested them in retrospective and prospective seroepidemiologic surveys. In contrast to the anticipated normal 50% seropositivity rate for IgG to B19 parvovirus in a population, none of the p donors

had evidence of previous infection. Two Tja- individuals traveled to the NIH to contribute bone marrow for research. In tissue culture, their marrow was not susceptible to parvovirus infection, and erythroid colony formation was normal. This is the first demonstration in humans of insusceptibility to a virus based on the genetic absence of the receptor. This discovery has implications for development of practical methods to remove parvovirus from biological samples, as, for example, from collections of pooled plasma, and for purification of parvovirus in the laboratory. In addition, the identification of the receptor has pathophysiologic implications for the development of vasculitis, myocarditis, and hemolytic anemia in the context of parvovirus infection.

In collaboration with Michael Rossman's laboratory at Purdue University, we have achieved 8.8 Å resolution of the atomic structure of empty parvovirus capsids by x-ray crystallography. By comparison with the structure of canine parvovirus, two important conclusions have been drawn. First, B19 shares with other icosahedral viruses an internal core composed of eight antiparallel beta-barrels. In contrast to canine parvovirus, there is no prominent surface spike at the 3-fold axis of symmetry. This spike is the site of antibody binding and tissue tropic amino acid residues in the animal parvoviruses, and the absence of this prominent landmark is consistent with marked differences in the immune response to human parvovirus epitopes.

Immunogenicity and Vaccine Development

Previously, we have shown that persistent infection in humans is the result of an inadequate antibody response. In particular, we showed that the antibody response to the minor capsid protein (VP1) was dominant. VP1 and VP2 are the two structural proteins of parvovirus, and they differ only in the additional 227 amino acids at the amino-terminus of VP1. VP1 is external to the capsid and contains neutralizing epitopes. We prepared 11 fusion peptides encompassing the entire structural protein sequence. These peptides were used to immunize animals, and the sera were then analyzed for virus binding and neutralizing activity. Almost all peptides showed good binding to virus, with the strongest activity located in the common region of VP1 and VP2. Surprisingly, neutralizing activity was present only in the unique region of VP1 and at the VP1-VP2 junction. We interpret these results to indicate that linear epitopes are best presented by the VP1 unique region and that conformational epitopes are dominant for the remainder of the capsid.

With MedImmune, our CRADA partner, we have continued development of a human vaccine for B19 parvovirus. We have demonstrated that baculovirus-derived capsids enriched in VP1 elicit the most potent neutralizing activity in three species of animals. Dose ranging and adjuvant studies have been completed in preparation for human trials, anticipated to begin later this year.

Because VP1 is external to the capsid and not required for capsid formation, we have hypothesized that the unique region might be replaced with other,

heterologous peptides. Such chimeric capsids would be useful for presentation of antigens to the immune system or for ligand-directed delivery of genetic material in gene therapy. We have succeeded in replacing substantial portions of the unique region with sequences derived from the major neutralizing epitope of the envelop glycoprotein of HIV-1 and of the hemagglutinin protein of the influenza virus. Despite replacement of the unique region of VP1, stable capsids form. Unfortunately, we have not yet been able to demonstrate external presentation of these antigens or their ability to illicit neutralizing antibody responses. Recently, we have replaced the VP1 unique region with hen egg white lysozyme and achieved capsids containing specific enzymatic activity, as assayed on a membrane substrate.

Non-Structural Protein

The non-structural protein of B19 parvovirus subserves all of the needed viral replicative functions, and in addition it is the factor responsible for cell death. We have expressed non-structural protein gene in a baculovirus system and purified it to homogeneity using high pressure liquid chromatography. We have identified nuclear localization signals within the sequence using transfection experiments. In a related project, we have identified a region of the terminal repeat sequence of the virus as specifically binding protein from human nuclear extracts; this protein has been purified and subjected to amino acid analysis. The protein may represent a novel transcription factor.

Bone Marrow Failure Syndromes, Pathogenesis and Treatment

Aplastic anemia has clinical and laboratory features that suggest that viruses or drugs incite pathophysiologic immunologic responses. We have previously demonstrated the presence of activated cytotoxic lymphocytes that overexpress gamma-interferon in the blood of patients with this disease, and levels of these cells fall as patients recover with immunosuppressive therapy. Studies during the current year have focused on several aspects of this problem; 1) identification of immune abnormalities in the target organ, the bone marrow; 2) search for a DNA virus in fulminant hepatitis; 3) characterization of hematopoietic suppression in AIDS; 4) immunosuppressive therapy of patients with severe aplastic anemia; and 5) epidemiological studies of aplastic anemia in an endemic region.

Immune Dysfunction in Bone Marrow

We have used a modification of the gene amplification technique (reverse polymerase chain reaction) to determine gene expression by gamma-interferon and lymphotoxin genes in the bone marrow of aplastic anemia patients. These genes express lymphokines that are produced by activated T-cells in tissue culture and in disease. We have shown that gamma-interferon is not expressed in normal bone marrow or in patients who have received multiple transfusions or who have bone marrow failure from a wide variety of etiologies. However, gamma-interferon gene

expression is prevalent in patients with severe aplastic anemia on presentation, present in 80 percent prior to therapy. With treatment, gamma-interferon RNA levels decrease or disappear, whereas relapsed patients again show gamma-interferon gene expression. Interferon gene expression is more sensitively assayed in bone marrow than in blood. In a smaller group of patients, lymphotoxin gene expression has been analyzed. Lymphotoxin is expressed in normal bone marrow, but at 5-10 fold higher levels in patients with severe aplastic anemia on presentation. Lymphocyte phenotype has also been determined by flow cytometric analysis of bone marrow compared to blood. The bone marrow of patients with aplastic anemia is characterized by markedly increased numbers of activated cytotoxic lymphocytes and natural killer cells, and the blood of some patients contains increased levels of gamma-delta T cell receptor-bearing cells. Results of studies of lymphokine expression and lymphocyte phenotype are consistent with a broad immunological response to an antigen or an autoimmune pathophysiology local to the marrow.

A DNA virus in fulminant hepatitis or hepatitis/aplasia?

This year a new and powerful approach to the analysis of novel DNA sequences was described by Dr. Michael Wiggler at the Cold Spring Harbor Laboratories. We have used this technique, which is based on reduction of the complexity of the human genome and gene amplification techniques, to analyze appropriate tissue samples from patients with fulminant hepatitis. Previously, we had determined that some cases of both fulminant hepatitis and the hepatitis/aplasia syndrome were non-A, non-B and non-C by serologic and molecular biologic techniques. In assays of two liver samples from patients with fulminant hepatitis, and comparison with parental DNA, we were unable to detect novel DNA sequences. These results strongly suggest that a putative virus in these syndromes does not contain a DNA genome but is more likely RNA. We are currently using gene amplification techniques with universal or degenerate sequences to determine if either coronavirus or retrovirus sequences are present in patients with these diseases.

Patients with AIDS commonly develop hematologic abnormalities. We speculated that HIV-1 infection of macrophages would cause release of tumor necrosis factor-alpha, a known inhibitor of hematopoiesis. Experiments in our laboratory have shown that TNF-alpha is released from bone marrow and blood macrophages on exposure to HIV-1 or purified envelop glycoprotein. Inhibition of hematopoiesis in these culture systems can be blocked by either antibodies to envelope glycoprotein or to TNF- α . We have been unable to detect direct infection of CD34+ hematopoietic progenitor cells, implicating an indirect mechanism for hematopoietic suppression in AIDS.

Immunosuppressive Therapy of Aplastic Anemia

We have continued to enroll patients in a protocol of intensive immunosuppression, consisting of antithymocyte globulin (ATG), cyclosporin, and methylprednisolone. This protocol has been extremely effective in patients with severe aplastic anemia, resulting in 70 percent hematologic remission rates at 1 year post-therapy. Fatalities have occurred only in patients with established fungal disease. Of particular note, both children and patients with absolute neutropenia have responded well to treatment. Combined immunosuppression offers a clearly dramatic improvement over the results in such patients with ATG alone.

Less successful is our effort to expand our therapeutic success using interleukin-3 in patients with Diamond-Blackfan syndrome. While this therapy was successful in causing long-term remissions in 2 of 6 cases in a pilot project, none of 17 patients treated with IL-3 alone have shown a significant response. These results suggest either that the IL-3 provided by Sandoz differs from the Immunex material (used in the original study) or that previous treatment with GM-CSF (as in the pilot study) is required.

Epidemiology of Aplastic Anemia

The Branch has continued its successful collaboration with colleagues in Thailand to complete a long-term epidemiologic survey of aplastic anemia and agranulocytosis in the Far East. Although data collection continues, early results are remarkable for demonstration of a very high incidence of the disease in Bangkok, an inverse correlation with socioeconomic status, but very little relationship to a prior drug use or pesticide exposure. These results are consistent with an infectious etiology for aplastic anemia in the Orient. This study has been expanded to include rural provinces of Thailand. In addition, contacts have been established in Vietnam, where a high rate of aplastic anemia is also suspected. There, hepatitis C virus appears to be endemic among blood donors. If political circumstances mature, formal collaborations between NHLBI and the Blood Transfusion Center in Saigon are planned.

Gene Therapy

Fanconi's Anemia

Fanconi's anemia is an autosomal recessive genetic disorder that produces aplastic anemia. Some but not all patients suffer congenital abnormalities, and there is a general predisposition to malignancies. The treatment of Fanconi's anemia is unsatisfying, as the only curative therapy is bone marrow transplantation, which has a significant risk of early mortality and is applicable only to patients with matched sibling donors.

There are at least four subtypes of Fanconi's anemia, as determined by complementation analysis. Recently, the gene responsible for Fanconi's anemia type C has been molecularly cloned. In collaboration with the discoverer of the gene, we have instigated a program for gene therapy of this disorder. The sequence for the gene has been incorporated into retrovirus and adenoassociated virus vectors. Both vectors are able to specifically reverse the Fanconi's phenotype in cell lines, in that they increase cell proliferation and resistance to clastogenic agents like mitomycin C. The vectors have similar and marked effects on CD34 + hematopoietic progenitors cells from patients, dramatically increasing colony formation alone and in the presence clastogenic agents. Protocols for the use of these vectors in patients are being developed.

A problem with gene therapy in Fanconi's anemia is the paucity of hematopoietic stem cells available for transfection. To circumvent this problem, we have instituted a clinical protocol for the treatment of patients with a hematopoietic growth factor, granulocyte colony stimulating factor (G-CSF), in order to increase circulating CD34 cells. The factor also has the potential benefit of increasing neutrophil numbers in severely neutropenic patients.

As part of clinical protocols in autologous bone marrow transplantation for multiple myeloma and chronic myelogenous leukemia, gene marking studies of hematopoietic cells have been initiated. Approved retroviral vectors containing different molecular versions of the neomycin resistance gene have been used to label CD34 + cells from peripheral blood and bone marrow in multiple myeloma and chronic leukemia, in order to determine whether relapse occurs as a result of inoculated cells. Similar studies are planned in aplastic anemia and other diseases to measure the kinetics of short and long-term repopulation from physically and/or phenotypically defined populations. Finally, basic laboratory work has focused on the relationship of specific growth factors and their receptors to malignant lymphocyte and plasma cell growth in myeloma. Antisense strategies in mouse models will help to define particularly the role of interleukin-3 and interleukin-6, and their receptors and receptor components in the regulation of the propagation of these cells. Insights gained from such studies may prove helpful in gene therapy approaches to these diseases.

ANNUAL REPORT OF THE HYPERTENSION-ENDOCRINE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1992 through September 30, 1993

The Hypertension-Endocrine Branch pursues studies into the mechanisms of blood pressure regulation and the causes and treatment of hypertension. Our activities have been concentrated in three areas: 1) vasoactive substances, 2) cardiac hypertrophy, and 3) pheochromocytoma.

I. Vasoactive substances. Atrial natriuretic peptide (ANP) (99-126) is a natriuretic peptide produced in the heart of most mammals. We performed studies to determine the action of ANP in heart failure and why the peptide is not more effective in reducing the edema of congestive heart failure (CHF). We found that the administration of ANP to control rats produced dose-dependent increases in urine flow, sodium excretion, and fractional sodium excretion. However, in rats, in which CHF was produced by creation of an aortocaval fistula, a different pattern was noted. In these rats, urinary sodium excretion is markedly decreased immediately after creation of the fistula. Sodium excretion in half of the animals soon returns to normal and they enter a stage of compensated CHF. However, the other animals are unable to excrete their sodium load, rapidly accumulate edema and die, if not treated appropriately (decompensated CHF). In rats with compensated CHF, the baseline values of fractional sodium excretion and glomerular filtration rate were significantly lower than in control rats and these parameters were decreased even further in rats with decompensated CHF. When equal doses of ANP were administered to these two groups of rats, the responses seen in control animals were decreased an average of 34% in rats with compensated CHF, but an average of 97% in animals with decompensated CHF. Chronic treatment with losartan, a potent inhibitor of angiotensin II receptors, resulted in dramatic natriuresis in animals with decompensated CHF. This confirms our hypothesis that activation of the renin-angiotensin-aldosterone system is the major cause of the failure to respond to either endogenous or exogenous ANF in CHF. In contrast, losartan treatment did not affect the daily sodium excretion in rats with either compensated CHF or control rats. In addition, when ANP was given to rats with decompensated CHF that had been pretreated with losartan, their natriuretic and diuretic responses to ANP improved significantly, nearly equal to the control animals.

In earlier studies, we had obtained similar results by blockade of the renin-angiotensin-aldosterone system with angiotensin-converting enzyme (ACE) inhibitors. The problem with those studies was that ACE inhibitors also inhibit bradykinin inactivation. The question still remained whether the improved natriuretic and diuretic responses might be due to the renal actions of bradykinin. Therefore, in another series of studies, we treated similar groups of rats with HOE-140, a potent bradykinin receptor antagonist. The use of this bradykinin antagonist did not affect the renal response to ANP in any of these groups of animals and, in addition, infusion of HOE-140 alone into rats with compensated CHF had no significant effect on the basal excretion of water or salt. Thus, these findings indicate that angiotensin II plays a major role in the development of sodium retention and in the blunted renal response to ANP in congestive heart failure and suggest that losartan is good therapy for cardiac edema. In addition, they show that bradykinin does not modulate the acute renal response to ANP in compensated CHF.

Endothelin (ET) is a powerful vasoconstrictor peptide, produced in vascular endothelium. ET is derived from a 38 amino acid precursor peptide, Big ET (BET). This cleavage to produce ET is performed by a still-unidentified protease, endothelin-converting enzyme (ECE). It has been suggested that neutral endopeptidase EC.3.4.24.11 converts BET to ET. This could be important since inhibitors of neutral endopeptidase already exist. We studied this conversion by examining the effects of recombinant neutral endopeptidase (rNEP) on the metabolism of I25I-ET-I. Incubation of ET-I with rNEP resulted in degradation of the peptide within minutes, whereas, its incubation with a ten-fold larger dose of the enzyme resulted in total cleavage within seconds. Both phosphoramidon and SQ 28,603, a converting enzyme inhibitor, fully protected ET-I from degradation by rNEP. The degradation of BET-I by rNEP was much slower than that of ET-I. Even after incubation with the larger concentration of enzyme, most of the radioactive counts in the sample eluted as intact peptide. Both phosphoramidon and SQ 28,603 prevented the degradation of BET by rNEP. In addition, at no time during the incubation of BET with rNEP was intact ET ever identified in the reaction mixture. Thus, it is clear that neutral endopeptidase EC.3.4.24.11 is not the endothelin-converting enzyme.

We also sought to determine if endothelium is required for the conversion of BET to ET by rings of aortic tissue. This was done in isolated rat aortic rings with and without endothelium. In this situation, both ET-I and BET-I caused dose-dependent contractions with no significant shift of the dose response curve. Thus, endothelium is not required for the vascular effects of either ET-I or BET-I, endothelium is not required for the conversion of BET to ET in aortic rings and ECE must exist in vascular smooth muscle.

II. Cardiac hypertrophy (CH) is one of the major consequences of sustained hypertension and is a major risk factor for cardiovascular morbidity and mortality. CH is induced by a wide variety of stimuli, including hemodynamic overload and neurohumoral and metabolic factors. We sought to determine whether the biochemical changes that accompany CH constitute an inherent feature of the hypertrophy, or alternately, whether these changes and cardiac hypertrophy are two different sets of adaptive stimuli that coincide in many cases. We also sought to assess the functional significance of the biochemical changes that accompany CH and to identify the mechanism that underlies the fundamental changes in gene expression in cardiac muscle during the development of hypertrophy.

We found that different patterns of CH are induced by different stimuli. These patterns are best exemplified by the comparison of CH induced by isoproterenol (ISO) and by phenylephrine (PE). ISO induces CH in all rats. The extent of ISO-CH decreases with age and is equal in males and females. In contrast, PE induces CH only in some animals without any correlation to changes in blood pressure. The percentage of animals that develop CH in response to PE increases with age and is greater in males than in females. ISO-CH develops rapidly (within days) and regresses quickly after cessation of the stimulus. PE-CH is slow to develop and to regress. Biochemically, PE-CH is characterized by an increased ratio of beta-myosin heavy chain (beta-MHC) to alpha-MHC, whereas ISO-CH presents with a decrease in this ratio. The change in MHC-isoforms by PE occurs equally in animals that respond to PE with CH and in those that do not. This indicates that the MHC change and CH are independent adaptive processes. The motility of labeled actin filaments on myosin extracted from hearts of PE-treated rats was normal as determined by the in-vitro motility assay. In contrast, actin motility on myosin extracted from hearts of ISO-treated rats exhibited an increased cycling rate. Thus, we have shown that alpha- and beta-adrenergic stimuli induce distinctly different patterns of CH which

differ in the rates of induction and of reversal of CH; the susceptibility of populations to develop CH according to age and gender; the percentage of animals that develop CH; and the biochemical and functional changes that accompany CH.

We also found that the stimuli for the initiation and for the maintenance of CH are different. Thus, we could show that ISO administration produced CH, which could be blocked by propranolol, a beta-blocker. ISO-CH could not be blocked by inhibitors of the renin-angiotensin-aldosterone system, i.e., captopril or losartan. On the other hand, once CH had been produced by ISO administration, the rate of regression of the hypertrophy was unaffected by alpha- or beta-blockers, while it was significantly retarded by angiotensin II. This clearly shows that the effect of ISO treatment to produce CH is entirely separate from the maintenance of CH by angiotensin II. Using a technique of subtractive hybridization, we have isolated eleven clones that are differentially expressed in the hypertrophied heart induced by ISO, and seven clones that are differentially expressed in the hypertrophied heart induced by PHE. Four of these clones overlap, i.e., they are expressed in both patterns of CH. We will explore these clones to determine whether the genes are expressed in myocytes or in cardiac connective tissue and then determine their structure and significance.

III. Pheochromocytoma. We have continued our efforts to improve the diagnosis, localization, and treatment of pheochromocytoma or paraganglioma. We have completed and published the results of a study in which we evaluated 36 patients with clinically suspected functioning paragangliomas by means of computerized tomography (CT), magnetic resonance imaging (MRI), and ¹³¹I- MIBG scintigraphy. The patients were divided into two subgroups: group I (n=21) patients studied before surgery, mainly had benign adrenal disease; group II (n=15) patients studied after surgery, frequently had malignant or extra-adrenal tumors. In group I, CT and MRI were more sensitive (100% for both), than MIBG scintigraphy (82%), which, however, was the most specific (100%). In group II, MIBG and MRI imaging were more sensitive (83% for both) than CT (75%), but MIBG was again the most specific (100%). Thus, we showed that all three techniques were complementary in localizing paragangliomas, both preoperatively and postoperatively. MIBG is indicated for both groups, but, it is especially recommended for postsurgical patients with recurrence, in whom the disease is often malignant or extra-adrenal.

In collaboration with the Clinical Neuroscience Branch, NINDS, we have evaluated the clinical usefulness of a new technique for measuring metanephrine (MN) and normetanephrine (NMN) in blood by means of liquid chromatography and electrochemical detection. In preliminary studies, we have shown that plasma NMN concentrations were increased in all 23 patients with pheochromocytoma, whereas MN concentrations were increased in only 9 patients. We have seen patients with surgically-proven paragangliomas who had normal plasma catecholamines, but clearly increased plasma NMN. This is probably due to the fact that these o-methylated metabolites of catecholamines have a longer half-life in the blood than the catecholamines themselves. Our preliminary findings indicate that the measurement of plasma NMN concentrations may be a more accurate means for the detection of pheochromocytoma.

**ANNUAL REPORT OF THE
LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE
October 1, 1992 to September 30, 1993**

Our continuing goal is to analyze the function of the kidney as a basis for understanding its pathophysiology and treating its disorders. Since the formation of urine depends upon the transport of water and solutes by kidney tubules, understanding renal function requires analysis of these cellular processes and of their integration in the kidney. Therefore, we are studying transport by cells in general and kidney cells in particular, as well as the mechanisms, hormonal and other, that support and control transport and metabolism.

Studies in single renal tubules

A. Receptors and signalling mechanisms in inner medullary collecting duct (IMCD).

Regulation of transport in the collecting duct is mediated by vasopressin and other hormones and autocoids, whose receptors are located in the basolateral plasma membrane. Maeda, Han, Ecelbarger, and Knepper have used FURA-2 loaded micro-dissected tubules to investigate neurohypophyseal hormone receptors which mobilize intracellular calcium in the rat IMCD. The studies demonstrated that vasopressin and oxytocin mobilize intracellular calcium via two different receptors. The vasopressin receptor is V1-like in character, but differs pharmacologically from the V1a receptor (the predominant receptor expressed in liver and vascular smooth muscle). Measurements using reverse transcription/ polymerase chain reaction (RT-PCR) in single microdissection rat tubules (DiGiovanni, Lolait, and Knepper) reveal substantial levels of V1a receptor mRNA in the cortical collecting duct, but little or no expression in the IMCD. As expected, high levels of V2 receptor expression were found in collecting duct segments. V1a receptor mRNA localization by in situ hybridization in adult rat kidneys (Ostrowski, Young, Lolait, and Knepper) revealed labelling of vascular elements and pelvic smooth muscle with the antisense V1a probe, but no evidence of V1a expression in renal tubules including collecting duct. It is speculated that the V1-like receptor in the rat IMCD may be due to the expression of a previous unidentified V1 receptor isotype or to promiscuous coupling of the V2 receptor to G-proteins which activate phosphatidylinositol specific phospholipase C.

Ecelbarger, Maeda, and Knepper are also investigating other calcium mobilizing receptors in the rat terminal IMCD. Screening a large number of hormones and autocoids that may play roles in regulation of IMCD function reveal significant increases in intracellular calcium in response to PGE₂, epidermal growth factor, carbachol, and ATP. The carbachol response was blocked by atropine, indicating a muscarinic mechanism. In contrast to the other agents, the ATP response is inhibited by extracellular calcium removal, suggesting that the rise may be due to calcium entry from the extracellular fluid. Extensive studies to characterize the

ATP receptor indicates that it is a non-selective "nucleotide receptor" rather than a P₂ purinergic receptor.

2. Mechanism of transport regulation in inner medullary collecting duct (IMCD).

Urea and water permeability are regulated by vasopressin in the IMCD. Increases in permeability are due to increases in the number of functional urea carriers and water channels in the apical membrane. In collaboration with Hediger and Smith who have cloned the urea carrier cDNA from rabbit kidney, DiGiovanni and Knepper have raised antibodies against synthetic peptides based on the cloned sequence. These antibodies are being used by Nielsen and Knepper to determine the cellular and subcellular localization of the urea carrier in rabbit and rat kidneys using immunocytochemical techniques.

DiGiovanni and Knepper have raised an antibody in rabbits to a synthetic peptide whose sequence is based on the cloned sequence of the rat vasopressin-regulated water channel protein. Immunocytochemical studies in rat kidneys by Nielsen and Knepper have demonstrated that the vasopressin-regulated water channel is confined to collecting ducts. Although it is found predominantly in the apical membrane, basolateral membrane labelling is seen also in the outer medullary and inner medullary collecting ducts. Immunocytochemistry at an electron microscopic level shows heavy labelling of subapical vesicles, which are proposed to be a reservoir of water channels that can be inserted into the apical membrane by regulated exocytosis.

Nielsen, Wall, Han, and Knepper have investigated the time course of vasopressin action in isolated perfused rat terminal IMCD segments. The major increases in water and urea permeability in response to vasopressin occur within 5-10 minutes of vasopressin addition. Similarly, the major decreases in permeabilities occur within 5 minutes of vasopressin withdrawal. Morphological studies in isolated perfused collecting ducts by Nielsen, Muller, and Knepper demonstrated very rapid internalization of luminal fluid-phase markers that occurs chiefly within 5 minutes of vasopressin withdrawal, consistent with the time course of permeability reversal. Fluid-phase markers were seen largely within small endosomes and small multivesicular bodies in the first 5 minutes of vasopressin withdrawal, but were seen predominantly in large multivesicular bodies beyond 5 minutes, indicating very rapid processing of endosomes. Whether these endosomes contain water channels will be investigated using the water channel antibody.

Mathematical modelling studies were carried out by Nielsen and Knepper to assess mechanisms of vasopressin-mediated regulation of water permeability. Conclusions were based on comparisons between the predicted dynamics of permeability changes and the measured time courses of water permeability in response to vasopressin addition and withdrawal. These studies show that the experimental data can be reproduced by the simulations only when it is assumed that both endo- and exocytosis are regulated by vasopressin.

In previous studies we have demonstrated that the vasopressin-independent water permeability of IMCD segments is markedly increased when the rats are water-restricted for more than 24 hours. Wade, Coleman, Nielsen, and Knepper are investigating the mechanisms involved in this conditioning response using freeze fracture electron microscopy in tubules that have been perfused in vitro. We have found that both vasopressin-treatment in vitro and thirsting in vivo (without vasopressin-treatment in vitro) are associated with a marked increase in intra-membrane particles in the apical membrane of IMCD cells. These particles are proposed to contain vasopressin-regulated water channels. Immunocytochemistry using the anti-water channel antibody revealed a marked increase in labelling of the apical membrane and subapical vesicles in response to thirsting, consistent with the view that water-restriction increases the total number of water channels in IMCD cells.

Han, Maeda, Ecelbarger, and Knepper have used microdissected IMCDs from water-restricted rats to investigate the possibility that water permeability in the collecting duct can be regulated by mechanisms that are independent of vasopressin-associated signalling. These tubules had a high baseline water permeability in the absence of vasopressin as previously demonstrated. Activation of the phosphoinositide pathway by carbachol was associated with a decrease in the vasopressin-independent water permeability, an effect that was blocked by the protein kinase C inhibitor calphostin C. The protein kinase C activator PMA mimicked that action of carbachol. Thus, water permeability regulation by activators of the phosphoinositide pathway occurs by a mechanism that is independent of vasopressin.

3. Transport characteristics of loop of Henle segments.

Chou, Nielsen, and Knepper are investigating the transport and morphological properties of chinchilla loop of Henle segments. Isolated perfused descending limbs were divisible into three subsegments based on functional criteria. In perfused tubules fixed for electron microscopy, it was found that the first two descending limb segments had morphological characteristics similar to those previously described in other rodents (so-called types II and III epithelia). However, the third segment (the pre-bend long-loop descending limb) had unique characteristics not previously described in the literature. This third segment has a low water permeability which is associated with an absence of labelling by an antibody that recognizes the descending limb/proximal tubule type water channel. The significance of the measured descending limb properties are being investigated by Layton, Chou, and Knepper using a sophisticated mathematical model that is solved using supercomputer technology. The model simulations demonstrate that the measured permeability properties in the chinchilla descending limb are inconsistent with a purely passive process for concentrating the inner medullary tissue.

4. Regulation of ammoniagenic enzymes.

DiGiovanni, Madsen, and Knepper are investigating the regulation of the ammoniagenic enzymes phosphate-dependent glutaminase (PDG) and glutamate dehydrogenase (GDH) in the S-1 segment of the rat proximal tubule. Experiments in rats treated with acid-loading demonstrated a gradual increase in PDG over four days without a significant change in GDH. The rise in PDG correlated with an increase in ammoniagenic capacity of isolated tubules. Methods have been worked out for measurement of PDG mRNA levels in single S-1 tubules, which will be used for investigation of the role of changes in mRNA levels in the observed regulation of PDG enzyme activity.

5. Ammonium transport mechanisms in kidney.

Flessner, Mejia, and Knepper have completed studies of NH_3 and NH_4^+ transport in isolated perfused loop of Henle segments, the results of which support the feasibility of our proposed countercurrent model of ammonium concentration in the urine. Mejia and Knepper are developing a large scale model of acid-base transport in the kidney which will allow us to assess the role of this countercurrent mechanism in overall regulation of acid-base excretion in the kidney.

Transport in model epithelia

The regulation of ionic pathways by a variety of arachidonic acid metabolites was studied by Kersting and Spring. They characterized the changes in ion transport caused by inhibition of the production of arachidonic acid metabolites in cultured human pancreatic cells with and without the cystic fibrosis genetic defect. They were able to rectify the defect by treatment of the cells with inhibitors of the production of epoxygenase metabolites of arachidonic acid. These studies indicate an important role for arachidonic metabolites in the control of ion transport involved in cystic fibrosis. The arachidonic acid metabolic pathways were also shown to influence transepithelial fluid transport in a model epithelium, *Necturus* gallbladder, but not to affect cell volume regulation.

Chatton, Persson, Nitschke, and Spring have developed the optical microscopy instrumentation and methods to study the composition of the fluid filling the intercellular spaces between epithelial cells. They utilize cultured renal cells (MDCK as well as LLC-PK₁), grown on glass coverslips or permeable supports to measure the pH, Na, and Cl of the spaces between the cells and to determine the diffusion coefficient of fluorescent dyes across the tight junctions between the cells as well as within the intercellular spaces.

Napathorn and Spring have confirmed and extended previous studies on the mechanism of sorbitol release from cultured rabbit papillary renal epithelial cells. They showed that the efflux of sorbitol can be inhibited by specific proteases and transport inhibitors. They synthesized several analogues of sorbitol and determined their rate of transport under both isotonic and hypotonic conditions. These studies have yielded considerable information on the selectivity of the sorbitol transporter.

Organic osmolytes

Bacterial, plant, and invertebrate animal cells are known to accumulate compatible, osmotically active, organic intracellular solutes when their environment becomes hyperosmotic. These organic "osmolytes" help maintain the intracellular milieu because they do not perturb vital intracellular macromolecules, in contrast to sodium and potassium salts which in abnormally high concentrations do perturb macromolecules. Most mammalian body fluids are not normally hyperosmotic and the cells exposed to them do not normally express organic osmolytes. The exception is the renal inner medulla in which the interstitial fluid is hyperosmotic to a variable extent because of the renal concentrating mechanism. We identified large and variable amounts of organic osmolytes in rat and rabbit inner medullary cells, namely sorbitol, inositol, glycerophosphorylcholine (GPC), taurine, and betaine.

Control of the cellular accumulation of these osmolytes is most readily studied in tissue culture. We screened several renal cell lines in hyperosmotic media and found that cells that survived accumulated the same organic osmolytes previously found in intact renal medullas. These cell lines are now being studied in detail. The findings with regard to osmotic regulation of the individual organic osmolytes are, as follows:

1. Sorbitol accumulates in GRB-PAP1 cells by synthesis from glucose, catalyzed by aldose reductase. Hypertonicity increases aldose reductase gene transcription, mRNA abundance, and protein abundance. We have cloned the rabbit aldose reductase gene and are testing the 5' flanking region in a transient expression system to determine the molecular mechanism by which hypertonicity stimulates transcription of this gene.
2. Inositol accumulates in MDCK cells when medium osmolality is increased. The mechanism is increased transport into the cells from the medium. We cloned the cDNA for the renal cell inositol transporter by expression in toad oocytes. Using the cDNA as a probe, we find increased expression of the inositol transporter in MDCK cells exposed to hypertonic medium. Current studies are investigating the effect of accumulation of inositol and the other osmolytes on expression of the inositol transporter.
3. Betaine, like inositol, is accumulated by MDCK cells in hyperosmotic medium because of increased transport into the cells. We cloned the cDNA for the renal cell betaine transporter by expression in toad oocytes. Using the cDNA as a probe, we find increased expression of the betaine transporter in MDCK cells exposed to hypertonic medium. The cloned betaine transporter has a nucleotide sequence similar to several recently cloned rat brain transporters which constitute a new transporter family. Current studies are investigating the effect of accumulation of betaine and the other osmolytes on expression of the betaine transporter.

4. GPC. In contrast to the other osmolytes, GPC accumulation is triggered by high urea, as well as by high NaCl. Accumulation of GPC by MDCK cells in hyperosmotic medium is due to increased net synthesis. Choline is an essential precursor. The choline is incorporated into phosphatidylcholine, which is hydrolyzed to form GPC. Activities of the enzymes involved in GPC synthesis (phospholipase A₂) and degradation (GPC: choline diesterase) are being measured. Depending on the conditions, the phospholipase may be activated and/or the diesterase may be inhibited to increase GPC; effects of urea and NaCl differ. NaCl and urea added together inhibit GPC:choline diesterase. We have purified GPC: choline diesterase, and derived some of its amino acid sequence. Present work is aimed at cDNA cloning of this enzyme and preparation of antibodies against it as steps toward determining how hyperosmolality controls its activity.

5. Stress proteins. Aldose reductase and the transporters for betaine and inositol are induced by the stress of hypertonicity. Heat shock proteins are induced by elevated temperature and other stresses. We are studying the relation between these responses. Expression of mRNA for the heat shock protein, HSP70, is increased by hypertonicity, as well as by elevated temperature. The common signal appears to be damage to cellular proteins by temperature or tonicity. Accumulation of betaine attenuates both responses, presumably because of its known action to stabilize proteins. On the other hand, expression of the mRNA for the betaine transporter is not affected by elevated temperature, demonstrating that it is not a heat shock protein and preserving the distinction between these two classes of stress proteins.

Accumulation of organic osmolytes in response to osmotic shock is a basic biological phenomenon previously identified from bacteria to cells in lower vertebrates. The present recognition of its vital role in renal medulla is the first indication that it is more than a curiosity in mammalian cells. Possible disorders of this system have not yet been investigated, but there are a number of poorly understood diseases of the renal medulla that should be considered. Further, the aldose reductase system, whose function we are unravelling in the renal medulla, is implicated in complications of diabetes in eyes, nerves, and kidneys.

Annual Report
Laboratory of Molecular Cardiology
National Heart, Lung, and Blood Institute
October 1, 1992 through September 30, 1993

The Laboratory of Molecular Cardiology investigates the regulation, expression and function of contractile proteins in muscle and nonmuscle cells. We are particularly interested in the mechanisms responsible for regulating the contractile activity of smooth muscle and nonmuscle cells as well as the factors that govern the expression of the genes encoding the contractile proteins. In addition, we have initiated a program to study a particular set of homeobox and pou genes that play a role in the early development of *Drosophila* and mammalian embryos. The purpose of these studies is to understand the mechanism of action and regulation of the various genes involved in neuromuscular and cardiac development. By studying the genes, mRNA and proteins involved in these developmental and contractile processes, we hope to understand the mechanisms by which cells differentiate, alter their phenotype, migrate, change their shape, move their membrane receptors, secrete cellular products and proliferate. We plan to use this information to understand both normal and disease processes.

Below is a summary of the various projects under study during the past year. Of particular note during the past year was: 1) expression of a soluble, truncated form of chicken brain myosin which has a phosphorylation-dependent, actin-activated MgATPase activity and can propel myosin in an *in vitro* motility assay; 2) identification of a myosin heavy chain isoform in *Xenopus* XTC cells which can be phosphorylated by cyclin-p34^{cdc2} kinase both *in situ* and *in vitro*; 3) characterization of a number of mutated isoforms of the slow skeletal and cardiac myosin heavy chain from patients suffering from the genetic disease hypertrophic cardiomyopathy; 4) identification of KRP (kinase related protein) as a phosphoprotein with a potential for stabilizing myosin filaments in smooth muscle and nonmuscle cells; 5) characterization of the promoter region of the human nonmuscle myosin heavy chain-A gene and identification of a region which affects cell type-dependent expression of this gene at pretranslational and translational steps; 6) evidence that the amino-terminal segment of the NK-4 *Drosophila* homeobox gene product contains a transcriptional activator domain and that NK-4 is a positive regulator of NK-3 gene expression; 7) identification of novel muscle-specific enhancer elements in the proximal promoter region of the *Drosophila* NK-1 homeobox gene.

Growth and Differentiation of Smooth Muscle and Nonmuscle Cells (S. Kawamoto, Z01 HL 01665-18 MC). This study is aimed at understanding the regulatory mechanisms underlying the expression of nonmuscle myosin heavy chain genes (MHC), which are tightly associated with cell growth and differentiation. Approximately 60 kb of the human nonmuscle MHC-A gene was examined for cell type cis-regulatory elements using NIH 3T3 mouse fibroblasts and the C2 mouse myogenic cell line. Two fragments from the first intron of the gene of approximately

0.5 kb and 5.5 kb were found to cause a 3-fold increase in the transcriptional activity of a reporter gene in NIH 3T3 fibroblasts and in proliferating C2 myoblasts, but not in differentiated C2 myotubes. In addition, a proximal downstream sequence was found to cause activation of a reporter gene in a cell-dependent manner. These 196 nucleotides consist of 100 nucleotides from exon 1 and 96 nucleotides from intron 1. Although it activated a luciferase reporter gene 50-fold in NIH 3T3 cells and 5-fold in C2 myoblasts, no activation was observed in C2 myotubes. Moreover, this fragment failed to activate luciferase production when displaced from its original position, raising the possibility that the activation mechanism may be posttranscriptional. By use of both RNAase protection assay, as well as competitive PCR, the increase in luciferase mRNA amount, due to the proximal downstream region, was found to be only 5-fold in NIH 3T3 cells. Since the increase in luciferase mRNA was not as large as the changes seen in the luciferase activity (reflecting protein amounts), which were 15-fold, it appears that the proximal downstream region affects both pretranslational (transcription or RNase stability) and translational steps.

Role of Phosphorylation as a Regulatory Mechanism in Muscle Contraction (J.R. Sellers, E.V. Harvey, W. Anderson, Z01 HL 01786-14 MC). Kinase related protein (KRP), also called telokin, is identical to the carboxyl-terminal 20 kD of myosin light chain kinase. Work in this laboratory (in collaboration with D.M. Watterson and V. Shirinsky) has demonstrated that this protein binds to smooth muscle and nonmuscle myosin and may play a role in stabilizing myosin filaments. Unphosphorylated and dephosphorylated vertebrate smooth muscle and nonmuscle myosin filaments are depolymerized at physiological ionic strength by the addition of ATP. This solubilized version of myosin adopts a folded, monomeric conformation with its carboxyl-terminal tail folding back over the head region. KRP appears to prevent this depolymerization by binding to myosin, most likely in the neck region. Of interest is the finding that KRP binds to dephosphorylated or unphosphorylated smooth muscle and nonmuscle myosin, but not to phosphorylated smooth muscle or nonmuscle myosin or to skeletal muscle myosin.

Nerve-specific Expression of Nonmuscle Myosin Heavy Chains (K. Itoh, R.S. Adelstein, Z01 HL 04208-07 MC). Previous work from this laboratory has shown that the mRNA encoding the myosin heavy chain-B (MHC-B) isoform of brain and spinal cord contains insertions of 30 nucleotides and 63 nucleotides near to the ATP binding region and the actin binding region in the myosin head (Takahashi et al., J. Biol. Chem. 267: 17864, 1992). In this study, we screened six different human cancer cell lines using the reverse transcriptase-competitive PCR, in order to determine whether or not the mRNA of these cells contained insertions at the ATP and/or actin binding domains. We found that only neuroblastoma cell lines showed inserted mRNA encoding 10 amino acids near to the ATP binding domain and that none of the cell lines showed an inserted mRNA encoding 21 amino acids near to the actin binding domain. We then analyzed a rat pheochromocytoma cell line, PC12, and found that prior to stimulation with nerve growth factor (NGF), these cells showed no evidence for an insertion. However, following stimulation with NGF, the inserted nucleotides were spliced and the percent of the inserted mRNA compared to the noninserted

mRNA rose from 0 to 40% by the end of the second week. Concomitant with the expression of the inserted nucleotides encoding the 10 amino acids, the cells underwent massive neurite outgrowth. Following the cessation of NGF stimulation, the mRNA containing the inserted nucleotides disappeared, as did the neurites.

Myosin Phosphorylation and the Regulation of Contractile Activity (C.A. Kelley, R.S. Adelstein, A. Smith, W. Anderson, Z01 HL 04210-06 MC). Recently, an isoform of vertebrate nonmuscle myosin heavy chain-B (MHC-B) with an inserted sequence of 10 amino acids (Takahashi et al., J. Biol. Chem. 267: 17864, 1992) and isoforms of smooth muscle myosin heavy chain 204 and 200, with insertions of 7 amino acids (Kelley et al., J. Biol. Chem. 268: 12848, 1993) at a site near the ATP binding region in the myosin head have been reported. In smooth muscle, this insert occurs in chicken intestinal, but not in chicken aorta myosin. The presence of the insert correlates with the ability of intestinal myosin to propel actin filaments more rapidly in an *in vitro* motility assay than aorta myosin as well as with a higher actin-activated Mg-ATPase activity. In nonmuscle myosin, but not in smooth muscle myosin, the insert contains a consensus sequence for phosphorylation by cyclin-p34^{cdc2} kinase and/or mitogen-activated protein kinase. Using myosin immunoprecipitated from cultured *Xenopus* XTC cells, we found that cdc2 kinase phosphorylated MHC-B isoforms only. Amino acid sequence of a tryptic peptide generated from the MHC-B demonstrated that the site phosphorylated was analogous to the inserted region found in chicken and human brain MHC. Moreover, tryptic phosphopeptide maps of the MHC-B phosphorylated in intact *Xenopus* cells during log phase growth show a phosphopeptide which comigrates with the tryptic phosphopeptide obtained from the same MHC phosphorylated *in vitro* with cdc2 kinase. These studies suggest that the *Xenopus* XTC isoform may be phosphorylated *in vivo* by cdc2 kinase and, thus, that this myosin isoform may play a role in mitosis.

Myosin Phosphorylation in Human T-lymphocytes and Bovine Brain (M. Moussavi, R.S. Adelstein, W. Anderson, Z01 HL 04216-03 MC). The purpose of these studies is to compare the site(s) phosphorylated by protein kinase C using human T-lymphocyte myosin and bovine brain myosin as substrates. Bovine brain myosin is known to be enriched with the nonmuscle myosin heavy chain-B (MHC-B) isoform whereas human T-lymphocytes contain both MHC-A and MHC-B isoforms. Using phorbol esters, an agent known to activate protein kinase C, we demonstrated that a single myosin tryptic phosphopeptide can be identified following incubation of human T-lymphocytes with radioactive ³²P. Peptide mapping revealed that this phosphopeptide comigrated with a hexapeptide previously identified by us as originating from the tail portion of the human platelet MHC. When bovine brain myosin was purified, two different isoforms of MHC-B could be separated on 5% acrylamide SDS-PAGE. Phosphorylation of the faster migrating isoform of the MHC by protein kinase C and tryptic digestion resulted in a single major phosphopeptide that mapped to a similar location to the peptide found in human T-lymphocytes.

Structure and Function of Cardiac Myosin (G. Cuda, J.R. Sellers, E. Harvey, W. Anderson, Z01 HL 04217-03 MC). The purpose of this study is to determine the

biochemical and enzymatic activity of β -myosin purified from skeletal and cardiac muscle biopsies in individuals with β -myosin heavy chain (MHC) gene linked hypertrophic cardiomyopathy. Recently, we have reported that the mutant β -myosin is expressed together with the wild type myosin in both skeletal and cardiac muscles of patients with specific mutations in the β -myosin heavy chain gene (Cuda et al., J. Clin. Invest. 91: 2861, 1993). This study demonstrates that myosin isolated from both the cardiac and skeletal muscle of hypertrophic cardiomyopathy patients propels actin filaments at a significantly lower velocity than myosin isolated from normal control muscles. Indeed, some β -MHC mutations (162^{Tyr→Cys}, 403^{Arg→Gln}) lead to a dramatic decrease in the rate of translocation of the actin filaments and this can be correlated with the location in the MHC of the mutated amino acids, near to the ATP binding domain (Cys 162) and near to the actin binding domain (Gln 403).

Expression of Nonmuscle Myosin Isoforms in Eukaryotic Cells (M.A. Conti, Y.S. Kim, R.S. Adelstein, Z01 HL 04218-03 MC). In order to understand the function of the A and B isoforms of myosin as well as the function of the insertions in the head region of the B isoform, we have initiated experiments to overexpress the full-length human nonmuscle myosin heavy chain (MHC) isoforms in a variety of mammalian cells. cDNA clones encoding the entire nonmuscle MHC-B will be introduced into a mammalian expression vector under the constitutive control of the Rous Sarcoma virus promoter. Constructs that contain the 20 kD and 17 kD myosin light chain have also been inserted in the same expression vector. Our plan is to introduce the cDNA encoding both the MHCs and MLCs as well as the individual light chains into a variety of cells.

Expression and Site-directed Mutagenesis of Nonmuscle Myosin Heavy Chains (R.S. Adelstein, Y.A. Preston, M.D. Pato, J.R. Sellers, W. Anderson, Z01 HL 04219-03 MC). Previous work from this laboratory has demonstrated that chicken and human brain tissue contain isoforms of nonmuscle myosin heavy chain-B (MHC-B) that contain mRNA encoding amino acids that are inserted into the ATP binding domain and actin binding domain in the head of the myosin heavy chain (Takahashi et al., J. Biol. Chem. 267: 17864, 1992). The 10 amino acids inserted near to the ATP binding site contain a putative sequence for cdc2 kinase and/or MAP kinase. Therefore, we undertook to express cDNA encoding both the inserted and noninserted isoforms of the MHC in order to study differences in their biological properties. To date, we have succeeded in expressing a truncated form of the nonmuscle MHC containing a 150 kD MHC as well as a full-length 20 kD and 17 kD myosin light chain. This myosin has the ability to propel actin filaments in an *in vitro* motility assay and to be activated by actin to hydrolyze Mg-ATP. Recently, we have succeeded in expressing the MHC that contains an inserted sequence of 10 amino acids near to the ATP binding site. Studies are presently underway to see whether the putative cdc2 and/or MAP kinase site can be phosphorylated *in vitro* and what effect the phosphorylation will have on myosin activity.

Identification of Chicken Pou Genes (M.B. Goens, Y.S. Kim, R.S. Adelstein, Z01 HL 04220-02 MC). Pou domain proteins are DNA binding proteins that control the

transcription of other genes, particularly during early development. Their control has been found to be important in the development of numerous systems including the anterior pituitary and the epidermis. We have identified a fragment of the pou gene by using the PCR and reverse transcription of mRNA from embryonic chicken heart primed with oligonucleotides generated on the basis of a known Drosophila pou gene nucleotide sequence. The sequence analysis of the cloned chicken pou gene fragment shows that it is related to other pou genes, but is divergent enough to suggest that it belongs to a novel class. The cDNA fragment that we isolated was used to probe a genomic library. Several genomic clones were isolated and are presently being characterized by restriction mapping and sequencing.

Function of Drosophila NK-homeobox Genes in Mesodermal Cell Differentiation (Y.S. Kim, Y.M. Lee, Z01 HL 04221-02 MC). The purpose of these studies is to establish the functional role of Drosophila NK-homeobox genes in mesodermal cell differentiation during embryogenesis. Genetic analysis has shown that two zygotic loci, twist and snail, are required for the formation of the mesoderm. The genes twist and snail are expressed in ventral cells including all future mesodermal cells. Previously, a novel homeobox gene cluster in the Drosophila at 93E1-5 region of the third chromosome was discovered (Kim and Nirenberg, PNAS 86: 7616, 1989). This locus includes the genes NK-1, NK-3, NK-4, Nkch4 and 93Ba1. The expression pattern of NK-4 and NK-3 homeobox genes together with that of the twist gene during embryogenesis suggested that these genes may regulate each other and, thereby, may control mesodermal cell differentiation. Studies in this laboratory have shown that the upstream region of NK-4 contains clusters of E-boxes which are known binding sites for HLH protein such as MyoD and twist.

cDNA Cloning and Characterization of Nonmuscle Myosin From Xenopus Laevis (N. Bhatia-Dey, R.S. Adelstein, Z01 HL 04222-02 MC). This study is aimed at characterizing the role of the forkhead domain containing genes in muscle, using Xenopus Laevis as a model system. A cDNA encoding the complete open reading frame of a somite-specific forkhead gene has been cloned from a Xenopus library and sequenced. Using Northern blot analysis, a 2.3 kb transcript was detected first at stage 10½ and is present until stage 40. The open reading frame shows a 77 amino acid forkhead domain which represents a putative DNA binding domain. Of particular note is the induction of the forkhead message by activin using an animal cap induction assay. Further experiments to characterize the expression of the protein encoded by this gene and to relate it to other muscle-specific transcription factors are presently underway.

Null Mutations of Vertebrate Myosin Heavy Chains (A.N. Tullio, R.S. Adelstein, Z01 HL 04223-01 MC). This research is directed at obtaining a transgenic mouse and an embryonic stem cell line that lacks one or both of the two nonmuscle myosin heavy chains (MHCs). Work in this laboratory has indicated that there are at least two genes that encode two different nonmuscle MHCs. In order to understand the function of the two isoforms, we are trying to knockout one of the two genes using homologous recombination in embryonic stem cells and, eventually, to create a

transgenic mouse which lacks one (or both) of the two isoforms. Therefore, a mouse genomic library was screened with a 5' human cDNA probe encoding nonmuscle MHC-A. One clone was isolated and partially characterized by restriction mapping and a fragment containing the ATG for the initiating methionine was identified. Similarly, a second mouse genomic library was screened with the 5' end of the cDNA clone encoding MHC-B and a genomic fragment containing the initiating methionine was subcloned into the Bluescript plasmid. Both mouse genomic fragments are being characterized by restriction mapping and will be altered by insertion of a neomycin encoding fragment prior to being introduced into mouse embryonic cells. This work is a collaboration with D. Accili (DB, NIDDK).

Function of NK-1 Homeobox Gene in Neuro-muscular Synaptogenesis (S.J. Kim, Y.S. Kim, Y.M. Lee, Z01 HL 04224-01 MC). The purpose of this research is to establish the functional role of the *Drosophila* NK-1 homeobox gene in neuro-muscular synaptogenesis. NK-1 is one of five homeobox genes clustered in the 93E1-5 region of the third chromosome of *Drosophila*. Characterization of the gene structure and *in situ* hybridization analysis suggests that these homeobox genes may be involved in mesodermal cell differentiation (NK-3 and NK-4) and muscle segment formation (NK-1). Previous data suggested that NK-1 may play an important role in neuro-muscular synaptogenesis and, as an initial step to understanding the activity-dependent regulation of the NK-1 homeobox in muscle segments and neurons, we analyzed the cis-acting DNA elements responsible for the expression of NK-1 in transfected myoblasts and neuroblastoma cell lines. From analysis of 4 kb of the 5' upstream region, it was shown that the region, comprised of 676 bp, had strong enhancer elements. Functional analysis of this region showed that an 86 bp DNA fragment was necessary and sufficient to demonstrate enhancer activity in C2C12 cells. However, additional regions were required for optimal enhancer activity in NG108-15 cells. We have also identified a strong silencer region. This negative element functions both in myoblast and neuroblastoma cell lines.

Regulatory Mechanisms for Nonmuscle Myosin Heavy Chain Gene Expression (K. Abe, S. Kawamoto, Z01 HL 04225-01 MC). The purpose of this study is to characterize human nonmuscle myosin heavy chain (MHC) genes and to understand the regulatory mechanism determining the expression of these genes. As an initial step in this undertaking, the promoter region of the human nonmuscle MHC-A gene has been characterized. The sequence of the promoter region showed many features typical of a housekeeping gene. For example, there is no TATA promoter element and the GC content is 77% in the 100 nucleotides upstream and downstream of the transcriptional start site. Moreover, multiple transcriptional start sites were observed in cells expressing this gene. Analysis of 5' deletion mutants of the promoter region revealed that an essential sequence for basal promoter activity resides in the 36 bp (from -78 to -113) upstream from the major transcriptional start site. Deletion of this sequence results in 80-90% loss of transcriptional activity. This region contains a single potential AP2 and SP1 binding site. Mutation of the AP2 site causes a 65% loss in promoter activity.

**MOLECULAR DISEASE BRANCH
NATIONAL HEART, LUNG, AND BLOOD INSTITUTE**

Cholesterol and triglycerides are transported in the blood by lipoproteins. Lipoproteins contain proteins, designated apolipoproteins (apo) as well as lipids including cholesterol, phospholipids and triglycerides. The overall objective of the combined clinical and basic research program of the Molecular Disease Branch is the elucidation of the role of plasma lipoproteins in lipid transport in normal individuals and in patients with elevated plasma levels of cholesterol and triglycerides who are at risk for the development of early heart disease or other diseases including pancreatitis. A major focus of research in the Branch is the analysis of the physiological role of apolipoproteins and lipoproteins in cholesterol and triglyceride transport, and the elucidation of the pathophysiological mechanisms involved in the regulation of lipoprotein biosynthesis, transport, and catabolism in normal subjects and patients with disorders of lipoprotein metabolism and atherosclerosis. Studies are also conducted to establish the genetic defects in patients with elevated blood lipids in order to establish improved methods for detection of individuals at risk for heart disease or pancreatitis.

Regulation Of Plasma Apolipoproteins Biosynthesis

The molecular mechanisms which modulate apolipoprotein gene expression are an active area of research and provide important information on lipoprotein metabolism. Research in the Branch has focused on apolipoprotein gene expression at the transcriptional and post-transcriptional levels.

A. Regulation of apoB biosynthesis

Initial studies on apolipoprotein gene expression focused on apoB, the major structural apolipoprotein on chylomicrons and VLDL. The cellular biosynthesis of apoB has been of particular interest since it provided insight into the mechanism for the biosynthesis of two plasma isoproteins from a single gene. The two B apolipoproteins, apoB-100 and apoB-48, are synthesized from a single apoB gene present on chromosome 2 by a mechanism which introduces a stop codon in the apoB mRNA. ApoB-100, the major apolipoprotein on LDL, contains 4536 amino acids and is translated from the full length 14.1 mRNA. A second mRNA is generated by a unique editing mechanism in which the CAA codon for the amino acid glutamine, residue 2253, is edited to a stop codon, UAA, which terminates synthesis of the B apolipoprotein. Translation of the edited mRNA results in the synthesis of apoB-48 which contains 2152 amino acids. In man, rat and rabbit intestinal apoB mRNA is approximately 85% edited and the major apoB isoprotein secreted is apoB-48. In the human and rabbit liver, apoB-100 is the primary isoprotein secreted. In contrast, in the rat approximately 65-70% of liver apoB is edited and both the B-48 and B-100 equivalent apolipoproteins are synthesized and secreted.

A series of studies have been performed to determine the time course and developmental signals that regulate liver and intestinal tissue specific expression of apoB editing. The percentage of apoB transcripts in the rat intestine which undergo editing significantly increases to adult levels the day prior to birth. However, the rat liver acquires adult editing capacity during the third post-natal week. In contrast to the rat, the small intestine of the human acquires 80 percent of adult levels of apoB mRNA by the end of the second trimester.

The thyroid hormone has been proposed to play a role in modulating apoB editing. To gain insight into the molecular mechanisms involved in this process human liver and intestinal cell lines were transfected with a plasmid encoding the thyroid nuclear receptor. Intestinal cells expressing the thyroid hormone receptor were treated with thyroid which resulted in a six-fold increase in the expression of the apoB mRNA editing process compared to either non-transfected or non-thyroid hormone treated control cells. The human liver cell line Hep-G2 is capable of editing 7% of mRNA transcripts with over-expression of the thyroid nuclear receptor. These studies provide the first insights into the molecular mechanism whereby thyroid hormone modulates apoB gene expression and the apoB mRNA editing process.

B. Post-translational modification of the apolipoproteins

The plasma apolipoproteins undergo post-translational modifications which may play an important role in apolipoprotein function. Modifications of the apolipoproteins thus far identified include phosphorylation, fatty acid acylation, and glycosylation. Phosphorylation has been shown to occur on apoA-I and apoB. The site of phosphorylation on apoA-I has been shown to occur on serine residue 201. Transfection studies using native apoA-I and apoA-I in which serine residue 201 was changed to alanine by site-directed mutagenesis established that phosphorylation was not required for biosynthesis and secretion of apoA-I from the cell. ApoA-I phosphorylation may

therefore play a role in intracellular trafficking of apoA-I. Studies on apoB revealed that the site of phosphorylation catalyzed by protein kinase C both in vitro and in vivo was on serine residues. Phosphorylation increased intracellular degradation of apoB indicating that phosphorylation may be an important determinant of the percentage of synthesized apoB which is secreted by the cell. O-glycosylation has been established as an important modulator of the distribution of apoA-II on plasma HDL. Glycosylated apoA-II did not associated with plasma lipoproteins, however, the non-glycosylated apoA-II was able to associated with all HDL subfractions. ApoA-I undergo fatty acid acylation. Evaluation of apoA-I by electrospray-mass spectrometry established that fatty acid acylation of plasma apoA-I is present only on proapoA-I and not mature apoA-I suggesting that acylation may be important in extracellular apoA-I metabolism. These combined results indicate that a variety of post-translational modifications of the apolipoproteins may play a central role in apolipoprotein biosynthesis, secretion, and catabolism.

C. Regulation of Lipoprotein Lipase And Its Cofactor ApoC-II

Lipoprotein lipase (LPL) is attached to the capillary endothelium and is the principal enzyme responsible for the hydrolysis of plasma triglycerides. An understanding of the structure and function of LPL is critical for our understanding of the biological function of this key enzyme in lipid and lipoprotein metabolism in normal subjects and in patients with hypertriglyceridemia. LPL, hepatic lipase (HL), and pancreatic lipase (PL) have a high degree of structural homology and form a lipase gene family. The crystalline structure of PL has been elucidated and used as a molecular model for the lipase gene family. Studies in the Branch have utilized site-directed mutagenesis and construction of chimeric enzymes to gain insight into the functional domains of LPL.

LPL contains a "lid" or loop of 22 amino acids from cysteine residue 216 to cysteine residue 239 which has been proposed to play an important role in modulating the access of lipid substrates to the catalytic site of the enzyme. Two amphipathic helices are present within this lid. In initial studies nine separate mutants were generated in which the amphipathic properties of the lid were altered. These studies established that disruption of the amphipathic helices abolished the ability of LPL to hydrolyze the emulsified substrate, triolein, but not the water soluble monodisperse substrate, tributyrin. These combined results indicate that the LPL lid containing the two amphipathic helices is essential for hydrolysis of emulsified, long chained fatty acid triglycerides and provide definitive evidence for the importance of the LPL lid in lipid-substrate interactions.

Recent studies have focused on the role of the LPL lid on substrate specificity. LPL containing the nine mutations and hepatic lipase (HL) have been analyzed as to their ability to hydrolyze different triglyceride (triolein) and phospholipid substrates. The two amphipathic helices in both LPL and HP are essential for phospholipid as well as triglyceride hydrolysis. The HL lid increases phospholipid hydrolysis whereas the LPL lid enhances triglyceride hydrolysis. These studies indicated that the lid may play a pivotal role in determining the substrate specificity of both LPL and HL.

ApoC-II is the principal cofactor required for the enzymic activity of LPL. Little definitive information is currently available regarding the mechanism(s) by which apoC-II increases the enzymic activity of LPL. The carboxyl (C)-terminal tetrapeptide of apoC-II (arginine-glycine-glutamic acid-glutamic acid) has been proposed to be essential for LPL activation by facilitating the ionic interaction between LPL and apoC-II. To test this hypothesis three mutations were introduced into apoC-II which altered the charge of the carboxyl-terminal tetrapeptide. These four mutations included 1. arginine-glycine-glutamic acid-glutamine, 2. arginine-glycine-glutamine-glutamine, 3. asparagine-glycine-glutamine-glutamine, and 4. Glutamine-glutamine-glutamine-glutamine. The specific activity of mutants 1,2,3, and 4 were 82, 30, 40, and 12 percent of normal apoC-II, respectively. Thus, elimination of 3 charged residues in the tetrapeptide did not prevent the activation of LPL thus establishing that ionic interaction of LPL with the charged tetrapeptide of apoC-II is not a prerequisite for LPL activation. However, elimination of the charges in the carboxyl-terminal tetrapeptide and the disruption of the predicted secondary structure of the carboxyl-terminal region of apoC-II as occurs in mutant 4 results in a virtually inactive apoC-II indicating that conformation may play a critical role in the apoC-II activation of LPL. These studies provide new interesting data on the mode of activation of LPL by apoC-II.

CLINICAL DISORDERS OF LIPOPROTEIN METABOLISM

Elevated blood levels of LDL and reduced levels of HDL have been well established as important risk factors for the development of early heart disease. Elevated levels of plasma triglycerides have been associated with both pancreatitis and early heart disease. A major focus of research in the Branch is a systematic analysis of triglyceride rich lipoproteins and HDL metabolism in normal subjects and in patients with both elevated and reduced levels of plasma triglycerides and HDL.

A. Triglyceride rich lipoprotein metabolism

1. Hepatic Lipase

Hepatic lipase plays an important role in HDL and intermediate density lipoprotein (IDL) metabolism. The major substrate for hepatic lipase is phospholipids, however, triglyceride hydrolysis is also catalyzed by hepatic lipase. The activity of hepatic lipase is increased by apoA-II and the major HDL subfraction containing hepatic lipase is LpA-I:A-II in HDL₂. HDL₂ are converted to HDL₃ and IDL are catabolized to LDL by the action of hepatic lipase. The increase in IDL is associated with an increased risk of premature cardiovascular disease.

A proband with a deficiency of hepatic lipase has been identified. The proband presented with a pseudo type III hyperlipoproteinemia including hypertriglyceridemia, pancreatitis, an increase in plasma β -VLDL as well as HDL₂, and early heart disease. Post-heparin hepatic lipase activity was not detectable. Structural analysis of the hepatic lipase gene in the proband revealed a single A-G mutation in intron 1 located within a potential branch lariat signal. This mutation generates an alternative splice site leading to the synthesis of a truncated, non-functional hepatic lipase enzyme. These results establish the importance of this sequence for normal splicing of hepatic lipase mRNA and identify alternative splicing as a novel mechanism leading to hepatic enzyme deficiency. There is only one previous kindred in the literature in which the genetic defect in the hepatic lipase gene has been determined in a proband with hepatic lipase deficiency.

The identification of this proband with hepatic lipase deficiency with an established molecular defect in the hepatic lipase gene will provide the opportunity to extend our knowledge of the role of hepatic lipase in lipoprotein metabolism and to determine the mechanism(s) responsible for the increased risk of premature cardiovascular disease in hepatic lipase deficiency.

2. Lipoprotein lipase

Over the last several years our Branch has elucidated the genetic defects in several kindreds with the familial hyperchylomicronemia syndrome. These studies have established the amino acid residues in the catalytic triad which are required for the activity of the enzyme and have identified the regions of the enzyme notably exon 5 which are required for lipoprotein lipase activity.

In the course of our studies on patients with the familial hyperchylomicronemia syndrome we have identified two kindreds which were unusual in their response to treatment. In these two probands their plasma triglycerides normalized after treatment with either omega-3-fatty acids or medium chain triglycerides (MCT) oil. Total LPL levels in both probands in post-heparin plasma samples were reduced to <10% of normal. Macrophage LDL mRNA levels and the specific activity of post-heparin plasma LPL were normal. The analysis of the lipoprotein lipase gene in both probands revealed no structural, regulatory, or splice defects. Thus, our studies have identified a subset of patients with unique genetic defects that lead to a functional deficiency of lipoprotein lipase and who respond to dietary oil therapy by normalizing triglycerides. Based on these results we propose that all patients presenting with apparent lipoprotein lipase deficiency be given a therapeutic trial of omega-3-fatty acids and MCT oil. The discovery of these two kindreds provides new insights into genetic heterogeneity of patients presenting with the familial chylomicronemia syndrome and provide evidence for two separate clinical variations of this syndrome. One cadre of patients have structural defects in the coding region of the lipoprotein lipase gene and do not normalize their triglyceride levels, while the other group of patients have low plasma levels of lipoprotein lipase following heparin injection however, there is no detectable defect in the lipoprotein lipase gene. The latter cadre of patients have a significant reduction in plasma triglycerides with dietary modification.

B. HDL metabolism

1. LpA-I and LpA-I:A-II metabolism in normal subjects

Plasma HDL are heterogeneous and different lipoprotein particles within HDL have been proposed to have separate physiological functions. Two important classes of lipoprotein particles within HDL are particles which contain both apoA-I and apoA-II (LpA-I:A-II), and particles which contain only apoA-I (LpA-I). LpA-I particles have been proposed to be the important anti-atherogenic particles within HDL which are involved in the removal of excess cholesterol from cells by a process which has been termed reverse cholesterol transport. LpA-I:A-II particles appear to offer little protection against the development of early heart disease. We have performed a series of detailed studies on LpA-I and LpA-I:A-II metabolism in normal subjects and subjects with reduced and elevated levels of HDL cholesterol. Detailed kinetic studies in normal volunteers established that LpA-I was catabolized at a significantly faster rate than LpA-I:A-II. There was also a net conversion of LpA-I to LpA-I:A-II.

In previous studies it was established that the most important metabolic determinant of plasma LpA-I levels is the rate of LpA-I catabolism. There are three major subclasses of LpA-I particles in HDL which have been designated Large, Medium and Small LpA-I. In vivo kinetics of these three particles revealed that the Small LpA-I particles are more rapidly catabolized than the Large and Medium particles. This is the first direct demonstration that the size of an HDL particle affects its catabolic rate. These combined results support the concept that LpA-I and LpA-I:A-II may have different metabolic and functional roles in lipoprotein metabolism.

2. Lecithin:cholesterol acyltransferase.

Lecithin cholesterol:acyltransferase (LCAT) is a plasma enzyme present primarily on HDL as well as LDL that is responsible for the esterification of virtually all free plasma cholesterol. Cholesteryl esters formed by LCAT are incorporated into the core of HDL and LDL particles. LCAT has been suggested to play a major role in reverse cholesterol transport with esterification of the cholesterol removed from cells and the transfer of the cholesteryl esters into the core of the lipoprotein particle for transport in plasma.

Patients with functional defects in LCAT present with two strikingly different clinical features. In Classical LCAT deficiency the patients have severe HDL deficiency, hemolytic anemia, cloudy corneas, and progressive renal disease. In a separate clinical syndrome, Fish Eye Disease (FED), the patients have HDL deficiency, severe cloudy corneas, but no renal disease or hemolytic anemia. During the last two years we have determined the LCAT enzymic activity and elucidated the molecular defect in the LCAT gene in probands with both Classical LCAT deficiency and FED. Based on these studies we have determined that the reason for the difference in the two clinical syndromes associated with LCAT deficiency is the residual activity of the mutant LCAT enzyme. Approximately 10 to 20% residual activity of the mutant LCAT enzyme appears to be sufficient activity to prevent the renal disease and hemolytic anemia.

Of particular interest in LCAT deficiency is the lack of premature cardiovascular disease despite the very low plasma HDL levels. Analysis of the plasma HDL revealed that there is a preferential decrease in the LpA-I:A-II particles with only a mild reduction in the LpA-I particles. Detailed kinetic studies using radiolabeled LpA-I and LpA-I:A-II established that LpA-I:A-II were catabolized faster than LpA-I resulting in a selective reduction in plasma LpA-I:A-II. These combined results indicate that the reduced plasma HDL in LCAT deficiency is due to increased catabolism. In addition, the preferential losses of the LpA-I:A-II particles but not the LpA-I particles provides an explanation for the lack of an increased risk of early heart disease in LCAT deficiency despite the very low plasma levels of HDL.

3. Cholesterol ester transfer protein (CETP)

CETP is present in normal plasma primarily in HDL on the LpA-I particles. CETP functions in lipoprotein metabolism in the exchange of cholesteryl esters and triglycerides between HDL and the apoB containing lipoproteins VLDL, IDL, and LDL. A deficiency of CETP is associated with hyperalphalipoproteinemia. All of the reported cases of CETP deficiency have been identified in Japan and have the same splice site mutation in the CETP gene. The plasma lipoproteins in CETP are quite unique and are characterized by markedly elevated plasma levels of HDL particles, reduced LDL and triglyceride enrichment of HDL as well as LDL. The elevated levels of HDL have been proposed to be protective and the subjects with CETP deficiency have been reported to have a longevity syndrome. Kinetic studies using radiolabeled lipoproteins as well as endogenous labelling with amino acids labelled with stable isotopes were performed in order to determine the metabolic mechanism responsible for the elevated levels of HDL. The catabolic rates of both LpA-I and LpA-I:A-II were delayed and the elevated plasma levels of HDL are due to decreased catabolism of the HDL particles. This is the first human dyslipoproteinemia which there is delayed catabolism leading to hyperalphalipoproteinemia. Kinetic analysis of the cholesteryl esters in HDL revealed that the cholesteryl esters are also markedly delayed in their removal from plasma. These results are of interest in that CETP deficiency is associated with elevated HDL levels however, the increased HDL level is due to decreased catabolism of both cholesteryl esters and HDL particles. These findings suggest that reverse cholesterol transport may actually be delayed in CETP deficiency, and that the high levels of HDL may not be protective in CETP deficiency.

In additional studies the rate of conversion of VLDL to LDL is significantly delayed in CETP deficiency resulting in markedly increased catabolism of VLDL before its conversion to LDL. These results account for the low plasma levels of apoB and LDL cholesterol. The combined results suggest that pharmacologic inhibition of CETP would be likely to delay catabolism of HDL and to cause metabolic channelling of VLDL to a degradation pathway rather than to LDL formation. Additional studies will be required to definitively establish if inhibition of CETP is a useful therapeutic approach to the treatment of premature cardiovascular disease.

4. Familial Hypoalphalipoproteinemia Syndrome

As previously reviewed decreased levels of HDL are inversely associated with the development of premature cardiovascular disease. However, within HDL the LpA-I but not the LpA-I:A-II are correlated with the risk of early heart disease. Patients have been identified that have low HDL as well as LpA-I levels that do not appear to be at risk for heart disease. We have identified five kindreds that have very low levels of HDL but have no family history of heart disease. Detailed kinetic studies were performed in these patients and the low HDL levels were shown to be due to a marked increased rate of catabolism of both LpA-I and LpA-I:A-II. Thus there are patients that have very low HDL levels due to increased catabolism of both LpA-I and LpA-I:A-II, however, no risk of heart disease is present in these kindreds. These results definitively establish that low plasma HDL levels may not predispose to early heart disease in every kindred with low HDL. Additional studies are currently in process to explore the metabolic mechanisms responsible for the increased catabolism of HDL in these kindreds.

C. Lp(a)

Lp(a) is an LDL-like lipoprotein which is associated with an increased risk of premature cardiovascular disease. The protein which is unique to Lp(a), apo(a), has a high degree of structural homology to plasminogen. Apo(a) is polymorphic, with many different sizes of this protein in the population. The size polymorphism is genetically determined. We have previously established that the different plasma levels of Lp(a) with the same molecular weight apo(a) isoform are due to variations in the production rate of Lp(a). More recently, a series of kinetic studies were carried out in individuals heterozygous for two isoforms of apo(a) and directly compared the metabolism of the two separate sized isoforms in the same individuals. The size of the apo(a) isoform does not affect its catabolic rate, but rather the rate of its production. These results established the metabolic basis for the association between apo(a) isoform size and plasma Lp(a) level, and direct attention to the mechanism by which the apo(a) size may modulate its rate of biosynthesis.

It has been controversial whether the LDL receptor pathway is the major site for catabolism of Lp(a) in vivo. To resolve this question kinetic studies were performed on Lp(a) in three patients with homozygous familial hypercholesterolemia (FH) who lack the LDL receptor. In all three patients, the catabolism of Lp(a) was similar to that in control subjects, indicating that the LDL receptor is not physiologically important for Lp(a) catabolism. In addition these studies revealed that the elevated plasma levels of Lp(a) in homozygous FH were due to increased production of Lp(a). Furthermore, in these studies we demonstrated for the first time that some Lp(a) is converted in vivo to LDL, providing important information about the metabolic pathways of Lp(a) in man.

Pathogenesis of Atherosclerosis

1. Transgenic rabbit model

A model system for familial hypercholesterolemia (FH) has been discovered in rabbits in which a partial deletion of a portion of the LDL receptor binding domain results in both profound hyperlipidemia and atherosclerosis. This strain of rabbit, termed the Watanabe Heritable Hyperlipoproteinemic (WHHL) rabbit, provides a means to test a variety of hypotheses relevant to lipoprotein metabolism and atherosclerosis. We have initiated the development of a transgenic rabbit program to systematically investigate the impact of genes relevant to HDL metabolism, reverse cholesterol transport, and atherosclerosis. This project was initiated by the cloning of genomic apolipoprotein A-I and the generation of a variety of constructs anticipated to induce over-expression of the linearized genomic construct. Superovulation strategies, mating and breeding techniques, and DNA as well as specific protein assays have all been developed over the past 2 years. We have evaluated the endocrinologic basis for infertility in WHHL rabbits and have characterized aberrant corpus luteal steroidogenesis present in WHHL rabbits. After refining our superovulation strategies, six transgenic rabbits have been successfully generated, three of which express apolipoprotein A-I. As little as 2 mg/dl of expression of human apolipoprotein A-I in the plasma of these rabbits have led to a 2-3 fold increase in the concentration of HDL cholesterol. We are currently characterizing the impact of over-expression of apolipoprotein A-I on both lipoprotein metabolism and on the ability of HDL to prevent atherosclerotic cardiovascular disease. The establishment of a transgenic rabbit program permits the direct testing of several central concepts in cardiovascular research including the reverse cholesterol transport hypothesis.

B. Post-prandial atherosclerosis

Epidemiologic studies have demonstrated correlations of dietary intake of total and saturated fats with the incidence of premature heart disease both within and between populations. However, only a small fraction of the population appear to be susceptible to diet-induced atherosclerosis. We have undertaken the investigation of human

intestinal apolipoprotein apoB editing as one potential molecular mechanism to explain this observation. As outlined above the human liver produces only apoB-100 and the human intestine synthesizes primarily apoB-48. We have previously established that 7% of the apoB synthesized by the human intestine is apoB-100. Since the plasma half-life of apoB-48 is <15 minutes and apoB-100 more than 2 days, production of enterocytically-derived apoB-100 could lead to triglyceride-rich atherogenic lipoprotein particles with a prolonged residence time in the blood the concentration of which could be influenced by diet. We evaluated the effect of a fat meal on human intestinal apoB synthesis utilizing intestinal biopsies obtained by endoscopy both before and after a fat meal. Pulse-chase labelling, immunoprecipitation, and sequential radioautography-Western blotting indicated that fat feeding did not change the relative amounts of apoB-100 synthesized in normolipidemic subjects. In contrast, patients with atherosclerosis, hypertriglyceridemia, and a family history of coronary artery disease showed changes in apoB mRNA editing. One proband was identified who synthesized 3 fold more apoB-100 than control subjects postprandially. This proband has been further investigated to determine the effect of loss of intestinal apoB mRNA editing on plasma lipoprotein metabolism. After a 3 kg weight loss and dietary modification, the proband with premature cardiovascular disease normalized his plasma triglyceride levels as well as the lipoprotein profile with concomitant normalization of his apoB mRNA editing. These findings suggest that diet-induced atherosclerosis can be due to defective intestinal apoB mRNA editing in some patients with premature coronary artery disease.

3. New non-invasive approach to the evaluation of coronary artery disease

One of the greatest challenges for the practicing physician is the evaluation of the patient for premature cardiovascular disease. Virtually no non-invasive techniques are available which definitively determine the presence and extent of cardiovascular disease in a patient at risk for the development of early heart disease. Patients homozygous for FH manifest profound hypercholesterolemia, cutaneous cholesterol deposits termed xanthomas, and cholesterol deposition in a variety of tissues including the eye, tendons, and inside the arterial vessels. These patients experience accelerated atherosclerosis and can manifest symptomatic cardiovascular disease from the ages of 2-30 years, and many die before the age of 20. The cause for the 10 fold increase in total and LDL cholesterol concentrations is a defect in the ability of the body to extract the cholesterol-rich LDL particles from the circulation via the LDL receptor pathway. A wide variety of mutations in the LDL receptor gene can lead to the loss of the expression of functional LDL receptors on the surface of liver cells. We have previously demonstrated that the degree of LDL receptor dysfunction on cultured skin fibroblasts from these patients highly correlates with the concentrations of LDL cholesterol present in their circulation. Over the years we have applied a variety of therapies to reduce the LDL cholesterol concentrations in these patients including diet, combination hypolipidemic drug therapy, portacaval shunting of the liver, plasma exchange, LDL apheresis, and liver transplantation. The degree of coronary artery atherosclerosis and the response to lipid-lowering intervention is variable among patients identified as having this disease. For the past 6 years we have prospectively evaluated the rate of progression of atherosclerosis by both invasive and non-invasive techniques in these patients. We have demonstrated that assessment of the extent and severity of atherosclerosis in these patients can be achieved using Ultrafast Computerized Axial Tomography. Not only was this non-invasive test useful in identifying atherosclerotic lesions in patients as young as 3 years of age, it also led to a new concept in atherosclerotic cardiovascular disease risk assessment, the cholesterol-years risk score. These findings in this inborn error in lipoprotein metabolism indicate that this new screening procedure will have important and practical applications for assessing cardiovascular risk in individuals with the more common forms of atherosclerosis.

Annual Report of the
Molecular Hematology Branch
National Heart, Lung, and Blood Institute
October 1, 1992 to September 30, 1993

This report covers the activities of the Sections on Molecular Genetics, Molecular Cloning, and the Section on RNA and Protein Biosynthesis. Increased emphasis has been directed towards the development of basic knowledge and technology required for the successful implementation of therapeutic concepts to human gene therapy.

SECTIONS ON MOLECULAR GENETICS AND MOLECULAR CLONING

Last year, several clinical gene therapy protocols were described for the treatment of adenosine deaminase deficiency and advanced cancer (malignant melanoma). This year, we have concentrated on the development of vectors and recombinant DNA technology for treatment of AIDS, hemophilia, intravascular thrombosis, as well as the use of genetically altered endothelial cells to improve the performance of vascular prosthetic devices.

During the past year, these Sections have achieved:

1. Retroviral vectors have been constructed that express SCD4, transdominant REV mutants, antisense RNA for TAR, and HIV inducible vectors for α -interferon, cytosine deaminase and diphtheria toxin.
2. It has been demonstrated that primary human T lymphocytes transduced with SCD4 vectors are protected from HIV infection in vitro.
3. A CD4-immunoglobulin fusion protein has been engineered to achieve a longer biological half-life.
4. The internal ribosomal entry site has been used in retroviral vectors to express combinations of rev plus SCD4 and SCD4 plus interferon to increase anti-HIV activity.
5. Based on research on various murine leukemia virus enhancer/promoter elements, a new vector has been constructed which expresses low levels of factor IX in T and B cell lines, as well as primary lymphocytes and CD34-enriched bone marrow.

6. Factor IX engineered cells have been grown in selectivity permeable membrane devices which allow the secretion of biologically active molecules to pass the membrane barrier.
7. A new retroviral vector for the expression of factor VIII from a spliced mRNA has been developed.
8. Overexpression of tissue plasminogen activator and urokinase using engineered retroviral vectors results in enhanced endothelial cell fibrinolytic activity; this led to significant prosthetic graft thrombolysis in a baboon model.
9. Viral vectors expressing recombinant biologically active hirudin (a specific thrombin inhibitor) are being tested for their ability to antagonize thrombin activation.
10. Very high levels of gene transfer into arterial endothelial cells have been achieved with adenoviral vectors.
11. Seeded vascular stents and grafts have been successfully implanted in vivo.

SECTION ON RNA AND PROTEIN BIOSYNTHESIS

To examine the mechanism(s) by which gene expression is regulated, we have characterized promoter elements of the genes encoding the alpha and beta subunits of the translation initiation factor eIF-2. In addition, we are studying the molecular basis of adeno-associated virus (AAV) integration into a unique site on the human chromosome 19, as well as a detailed biochemical examination of the AAV Rep protein functions. Factors affecting the efficiency and specificity of homologous recombination in human cell lines have also been determined.

During the past year the section has achieved the following major goals.

1. Two novel promoter elements of the eIF-2 α gene were shown to regulate expression by the generation of overlapping antisense transcripts.
2. The eukaryotic transcription factor α -PAL that regulates the rate of sense eIF-2 α transcription, has been purified to homogeneity, cloned and sequenced.
3. The full length eIF-2 β gene has been cloned and shown to also have an α -PAL binding site similar to eIF-2 α .

4. The 12 bp consensus recognition sequence for α -PAL was deduced by selection from a random oligonucleotide library. Over 40 genes concerned with cell growth appear to utilize the factor to regulate their expression.
5. The frequency of homologous recombination has been increased by several orders of magnitude by optimization of electroporation parameters.
6. The AAV Rep 68 and 78 proteins have been overexpressed in *E. coli* as MBP-Rep fusion proteins and purified to homogeneity. All in vitro activities of the wt Rep proteins are conserved.
7. The 1.6 and 4.5 kb eIF-2 α cDNAs have been fully sequenced and shown to arise from alternate polyadenylation site selection.
8. The 20-fold increase in translational activity that occurs within the first 24 hrs of mitogenic stimulation of T lymphocytes has been shown to result from increased (50-100 fold) translation factor expression, in addition to activation of eIF-4 activity by phosphorylation of its α subunit.
9. Coordinate synthesis of the eIF-2 α and β subunits is achieved by a novel regulation of the rate of elongation.

Annual Report of the Pathology Branch
Division of Intramural Research
National Heart, Lung, and Blood Institute
October 1, 1992, to September 30, 1993

The Pathology Branch maintains a broad range of research interests in Cardiovascular and Pulmonary Pathology. During the past year, research studies were completed on different aspects of infective endocarditis, myocardial diseases, and coronary artery disease in the elderly. In addition the Ultrastructure Section has completed studies on the cardiomyopathy associated with chronic Chagas' disease, the interstitial dendritic cells of the heart, the cardiotoxicity of antineoplastic agents, porcine aortic valvular bioprostheses, human cardiac valvular allografts and the prevention of lesions induced by hyperoxia.

Studies on Infective Endocarditis

Infective Endocarditis of the Mitral Valve

Morphologic studies were made of the hearts of 63 patients with active infective endocarditis of the mitral valve. These studies demonstrated preexisting anatomic abnormalities in 21 of the 63 cases. These abnormalities consisted of: rheumatic heart disease in 8, mitral valve prolapse in 3, hypertrophic cardiomyopathy in 3 and mitral annular calcification in 9. The remaining 42 patients were considered to have had anatomically normal valves prior to the onset of the infection. Twenty-two of the 42 patients with previously normal mitral valves had various factors predisposing to infection. This study provides a variety of clinicopathologic correlations in patients with endocarditis of the mitral valve, including predisposing factors and features related to the presence or absence of preexisting abnormalities of the mitral valve.

Endocarditis in Previously Non-Stenotic and Stenotic Aortic Valves

Clinical and cardiac necropsy findings were analyzed in 96 patients with active infective endocarditis involving the aortic valve. Twenty-five of these patients, (26%) had active infective endocarditis superimposed on a previously stenotic aortic valve and 71 (74%) on a previously nonstenotic aortic valve. The patients with stenotic aortic valves, compared to those with nonstenotic aortic valves had significantly higher mean ages, a higher percent over 60 years of age, a higher percent of men, a higher frequency of an absent or unknown predisposing factor to infection, a lower frequency of a precordial murmur of aortic regurgitation, a lower percent with a long duration (>60 days) of signs and symptoms indicative of infective endocarditis, a larger mean heart weight, a higher percent with aortic valve calcific deposits, and a higher frequency of associated ring abscess. Thus, active infective endocarditis superimposed on previously stenotic aortic valves differs in many significant respects from that which develops in previously nonstenotic aortic valves. The most important of these differences concerns the frequency of abscesses in the aortic valvular ring.

Infective Endocarditis of Bioprosthetic Heart Valves

The exact localization of the site of infection in endocarditis involving implanted bioprosthetic heart valves is of importance in the management of this complication. To assess this problem we studied 34 patients with active bioprosthetic infection at the time of valve replacement. In these 34 patients, 43 bioprostheses had been implanted and 37 were infected. Five patients had infection occurring 260 days after operation; the location of infection was the cusps only in 2, the ring only in 2 and both locations in 3. In 16 patients with isolated aortic valve replacement, 4 had ring infection only, 6 had cusp infection only and 6 had infection in both locations. In the 6 patients with isolated mitral valve replacement, 2 had cusp infection only, 1 had ring infection only and 3 had infection in both locations. In the remaining 7 patients, 15 native valves were replaced with bioprostheses and 10 of them were infected. The infection involved only the cusps in 7, and both cusps and ring in 3. Ring abscesses are very difficult to treat and are

associated with considerable damage to the tissues surrounding the bioprotheses, thus leading to considerable difficulty in the surgical implantation of a new valve.

Studies on Myocardial Diseases

Pathology of Acute Myocarditis in Patients with Sudden Deaths

The types and numbers of various infiltrating inflammatory cells in acute myocarditis have been the subject of numerous studies. However, no data are available on the topographic distribution of inflammatory cells in the hearts of patients with acute, fatal myocarditis. To evaluate this problem, we made studies of the gross myocardial lesions and the relative density of the inflammatory cells in various myocardial locations (subepicardial and subendocardial halves of the left ventricular free wall, right ventricular and left ventricular halves of the ventricular septum, and the right ventricular free wall) in 14 patients who died suddenly of acute mononuclear cell myocarditis. The highest mean number of mononuclear cells were seen in the subepicardial regions of the left ventricular free wall (193 cells per high power field) and the lowest in the right ventricular one half of the ventricular septum (92 cells per high power field, $p < 0.001$). Thus, this study shows that inflammatory cells in acute, fatal, mononuclear myocarditis are more numerous in the subepicardial than in the subendocardial zones of the ventricles. This observation is of practical importance in the interpretation of endomyocardial biopsies, most of which are obtained from the subendocardial region of the right side of the ventricular septum.

Subepicardial Myocardial Lesions

The spectrum of cardiac diseases associated with epicardial inflammatory and fibrosing lesions has not been adequately defined. To survey the range of diseases associated with such lesions, a retrospective review of the clinical and cardiac morphologic data was made in 22 patients who had lesions located in this site. These patients ranged in age from 14 to 73 years (mean 47), and 20 were men. The lesions were associated with atherosclerotic coronary artery disease in six patients, sarcoidosis in five, idiopathic dilated cardiomyopathy in four, lymphocytic myocarditis in two, and hypoplastic right and left circumflex coronary arteries in one. In four patients the cause was unclear. In the patients with atherosclerotic coronary artery disease, the subepicardial myocardial lesions were small, few in number, and located in the left ventricular posterior wall. In patients with sarcoidosis or myocarditis, the subepicardial lesions were extensive and commonly associated with transmural left and right ventricular lesions. The right ventricular half of the ventricular septum also was frequently affected. In the remaining nine patients, the subepicardial lesions were small and unassociated with transmural left ventricular lesions. Thus, subepicardial myocardial lesions are seen in a variety of cardiac diseases. The distribution of the gross lesions can assist in delineating the underlying disease process.

Cardiac Collagen Matrix in Hypertrophic Cardiomyopathy

The occurrence of myocardial fibrosis in patients with hypertrophic cardiomyopathy is well recognized, particularly in the asymmetrically hypertrophied ventricular septum. However, the extent of this fibrosis has not been quantitatively assessed. Sirius red staining (which has been previously shown to stain collagen in a highly selective manner) and videodensitometry were used to determine the concentration of total, interstitial and perivascular collagen in sections of ventricular septum from 16 patients with hypertrophic cardiomyopathy. The findings were compared to those in 16 normal hearts of patients who died of non-cardiac causes. The quantity of total collagen was increased 4-fold, and that of interstitial collagen 7-fold, in patients with hypertrophic cardiomyopathy compared to normal controls. In patients with hypertrophic cardiomyopathy, no correlation existed between age, ventricular septal thickness, degree of myofiber disarray and quantity of collagen. This poor correlation appears to be due to the multiplicity of factors that can induce myocardial fibrosis in patients with hypertrophic cardiomyopathy.

Lipomatous Hypertrophy of the Atrial Septum

The term lipomatous hypertrophy of the atrial septum refers to a poorly understood condition that is characterized by the accumulation of adipose tissue in the upper portion of the atrial septum. To study clinicopathologic correlations in patients with massive fatty deposits in the atrial septum, various clinical and pathologic parameters were analyzed in 80 necropsy patients in whom the thickness of the atrial septum cephalad to the fossa ovalis was equal to or greater than 2cm. The thickness of the atrial septum in the 80 patients correlated with body weight and with the thickness of the adipose tissue in the atrioventricular groove and that covering the right ventricle. In 53 patients (67%), one or more of the four major epicardial coronary arteries were narrowed >75% in cross-sectional area by atherosclerotic plaque. Atrial arrhythmias were present in 31 patients (40%). Patients with larger deposits of fat (atrial septal thickness ≥ 3 cm) had a higher frequency of atrial arrhythmias (60% vs. 34%, $p < 0.01$). The atrial septum was significantly thicker in patients with atrial arrhythmias compared with those without atrial arrhythmias (2.9 vs. 2.3 cm, $p < 0.01$). Of the 28 patients with available electrocardiograms, 20 (71%) showed atrial arrhythmias (atrial premature complexes in nine, atrial fibrillation in seven, atrial tachycardia in three, ectopic atrial rhythm in one and junctional rhythm in one). This study demonstrates that massive fatty deposits in the atrial septum are associated with large deposits of fat elsewhere in the body and in other parts of the heart. They are frequently associated with atrial arrhythmias and atherosclerotic coronary artery disease.

Studies on Aging and Coronary Artery Disease

Cardiac Morphologic Abnormalities in Octogenarians

Only limited information is available on the cardiovascular findings in individuals with very prolonged life spans. To study the morphologic abnormalities in the hearts of octogenarians, a review of the clinical data and necropsy findings was made in 366 patients ranging in age from 80-89 years at the time of death. They died of cardiac [195 (48%)], vascular [47 (13%)] or non-cardiovascular [125 (39%)] causes. Atherosclerotic coronary artery disease was present in 218 (60%, 111 men). A mean of 1.7, 1.3 and 0.7 of the 4 major epicardial coronary arteries were narrowed >75% in cross-sectional area by atherosclerotic plaque in those who died of cardiac, vascular and non-cardiovascular causes, respectively. Calcific deposits were present in the mitral annulus [140 (38%)], aortic valve cusps [153 (42%)] and coronary arteries [185 (78%)]. Among cardiac causes of death, coronary artery disease was the most common [127/195 (65%)], of which 87 (68%) was due to acute myocardial infarction. Significant cardiac morphologic differences were found between men and women who died of acute myocardial infarction. The present study demonstrates that coronary artery disease is a major cause of death in octogenarians. Compared to men, higher numbers of women have their first acute myocardial infarction at this age group and in them complications of the acute myocardial infarction are common.

ULTRASTRUCTURE SECTION

Studies on Chagas Diseases

Chagas Disease as an Undiagnosed Type of Cardiomyopathy in the U.S.A.

To evaluate the extent to which chronic Chagasic cardiomyopathy is present as an undiagnosed form of heart disease in the United States, a review was made of data pertinent to the occurrence and clinical features of this disease in Latin America and in the United States. In Latin America, 16 to 18 million individuals are thought to have Chagas' disease and 90 million are considered to be at risk of infection. In the U.S., the occurrence of Chagas' disease is virtually limited to individuals who have resided in Latin America, where they acquired the infection, and then migrated to this country. Data on the prevalence of positive serologic reactions for *Trypanosoma cruzi* serve for calculating that a total of up to 74,000 Latin Americans residing in the U.S. have the chronic form of chagasic cardiomyopathy. The vast majority of these individuals are either undiagnosed, or misdiagnosed as having idiopathic dilated cardiomyopathy or coronary artery disease. Vector transmission of *Trypanosoma cruzi* infection is very unlikely to occur in the U.S.

Transfusion of blood from infected but asymptomatic individuals is the most important mechanism of transmission of this disorder in the U.S.

Endomyocardial Biopsies in Chronic Chagasic Cardiomyopathy

Although the cardiac pathologic findings in patients with chronic Chagasic cardiomyopathy have been documented in hearts obtained at necropsy, only limited information is available on morphologic alterations evident on study of endomyocardial biopsies obtained from patients with earlier stages of the disease. To define the morphologic findings in myocardial biopsies from patients with chronic Chagasic cardiomyopathy, catheter biopsy specimens from 30 of these patients were examined using histologic immunohistochemical and electronmicroscopic methods. The biopsy specimens showed variable degrees of myocardial hypertrophy and mononuclear infiltrates. No tissue forms of trypanosomas were found. The endocardium averaged $24 \pm 12.6 \mu\text{m}$ (mean \pm SD) in thickness. The mean myocyte diameter was $20 \pm 7.33 \mu\text{m}$. The hearts were severely fibrotic, containing a mean of $24.1 \pm 12.8\%$ of fibrous tissue (range 8.2-49%); mast cells were scarce. The basement membranes of endothelial cells, vascular smooth muscle cells and cardiac myocytes frequently showed considerable thickening. Mononuclear cell infiltrates were found in 25 of the 30 biopsies. In 12 of these biopsies, immunocytochemical techniques were used to identify and count leukocytes, T lymphocytes and B lymphocytes. These studies showed that the majority of the lymphocytes were T lymphocytes and were associated with necrotic or degenerating myocytes. Other studies currently under way in our unit support the concept that most of the tissue damage in Chagasic myocarditis is due to cytotoxic and cytolytic effects mediated by contacts between lymphocytes and cardiac endothelial cells and myocytes.

Studies on Dendritic Cells of the Heart

Interstitial Dendritic Cells of the Heart in Myocardial Infarction

Interstitial dendritic cells are the most recently discovered cell type in myocardium, and very little is known of their role in cardiac diseases. These cells are important for the initiation of immune responses, as they function as antigen-presenting cells and have close interactions with lymphocytes and macrophages. We have been successful in applying monoclonal antibody techniques for the immunohistochemical identification of the interstitial dendritic cells of the heart. We have undertaken several studies designed to evaluate the structure and function of cardiac interstitial dendritic cells in a variety of pathological conditions. In the first of these studies, we have analyzed the qualitative and quantitative alterations that myocardial interstitial dendritic cells undergo in response to experimentally induced myocardial infarction (MI) in rats. Left coronary arterial ligations were performed in 43 Sprague-Dawley rats, which were sacrificed 2, 4, 7, 14 and 21 days after operation. Thirteen unoperated and 39 sham-operated rats were used as controls. Frozen sections were stained with monoclonal antibodies (OX 6 and W3/25) to identify and count interstitial dendritic cells. Immunoelectron microscopy was also used to identify interstitial dendritic cells by means of immunoperoxidase methods. The numbers of dendritic cells/mm² of tissue section were calculated for all hearts. In hearts with MI, dendritic cells were counted in three areas: the center of the MI, the border zone, and the noninfarcted left ventricle. The numbers of dendritic cells/mm² were 82 ± 10 in the left ventricle of unoperated rats. Hearts with MI showed marked increases in the numbers of dendritic cells/mm² in the border zone (796 ± 79 at 7 days and 528 ± 98 at 14 days). In the border zone, dendritic cells often were associated with small clusters of T-helper lymphocytes, which reacted with W3/25 antibody (the rat homolog of human CD4). The center of the MI showed an increase in dendritic cells only on day 7 (120 ± 18). By 21 days dendritic cells in the border zone were only slightly increased in number (159 ± 15). The findings of the present study indicate that dendritic cells (which are derived from precursor cells in bone marrow), migrate to the heart in response to antigenic materials released by necrotic cardiac muscle cells in myocardial infarction. These cells become concentrated in the border zone of the infarct and participate in the activation of lymphocytes and in the initiation of immune responses, and decrease in number as inflammation subsides and fibrous scarring develops.

Effects of Doxorubicin on Cardiac Dendritic Cells in Hypertensive Rats

An investigation was made using immunohistochemical and morphometric techniques, of the changes induced in the hearts of spontaneously hypertensive rats (SHR) by the administration of doxorubicin. This agent is well known to have cardiotoxic properties which result in dilated cardiomyopathy. It has been assumed that doxorubicin depresses immune reactions, but the influence of this agent on the immune effector cells of the heart has not been investigated. Therefore, these changes were studied using the SHR model that has been previously utilized to characterize extensively other aspects of doxorubicin cardiotoxicity. Histologic and immunohistochemical studies using specific monoclonal antibodies were made to evaluate the severity of the chronic cardiomyopathy and the quantitative changes in interstitial dendritic cells, T-helper lymphocytes, T-cytotoxic/suppressor lymphocytes and macrophages in the hearts of SHR treated with doxorubicin, 1 mg/kg per week for 3, 6, 9 or 12 weeks. In addition, an assessment was made of the modifications of the responses of these cell populations by pretreatment of the SHR with ICRF-187, which has been previously shown to protect against doxorubicin cardiotoxicity. The number of interstitial dendritic cells/mm² of section of left ventricle was similar in saline-treated control SHR and in those treated with ICRF-187 alone, but increased markedly in animals receiving a total cumulative dose of 12 mg/kg doxorubicin. Treatment with ICRF-187 prior to each administration of doxorubicin attenuated in a dose-dependent manner the increase in numbers of dendritic cells induced by doxorubicin. Doxorubicin also induced increases in the numbers of T-helper lymphocytes and macrophages, but not of T-cytotoxic/suppressor lymphocytes. These increases were also attenuated by pretreatment with ICRF-187. These data are consistent with the concept that doxorubicin cardiotoxicity results in the release of substances that initiate immune reactions and that such reactions are attenuated by pretreatment with ICRF-187.

Studies on Cardiotoxicity of Drugs

The Role of Myocardial Biopsy in the Diagnosis of Anthracycline Toxicity.

Light and electron microscopic examination of endomyocardial tissue obtained by catheter biopsy from the right side of the ventricular septum constitutes a useful and accurate technique for evaluating the degree of cardiac toxicity produced by the administration of antineoplastic agents of the anthracycline family. These agents produce characteristic myocardial lesions, in which two main components are recognizable: myofibrillar loss and cytoplasmic vacuolization due to dilatation of the sarcoplasmic reticulum. When toxicity is minimal, these changes affect only a small percentage of the myocytes; as the severity of the toxicity increases, a progressively larger percentage of myocytes is affected. Accordingly, a semi-quantitative scale (Billingham scale) has been devised in which these changes are graded from 0 to 3+. The grading score has been found to correlate with the total cumulative dose of the anthracycline and with the extent of cardiac functional impairment. High scores have been found to be associated with risk factors such as cumulative doses of anthracyclines in excess of 550 mg/m² of body surface, advanced age (> than 70 years of age), systemic arterial hypertension, presence of other cardiovascular diseases, and previous radiotherapy to the mediastinum. In patients with these risk factors, monitoring by means of cardiac biopsy is recommended to guide therapy with anthracyclines beyond the empirical dose limitations.

Pathologic Anatomy of Animal Models of Anthracycline Cardiotoxicity

An analysis of the anatomic findings in experimental animals treated with anthracyclines was made in order to select the most suitable animal model for the study of anthracycline-induced cardiomyopathy. The basic features of the cardiac lesions, i.e., dilatation of sarcoplasmic reticulum and myofibrillar loss, tend to have a similar occurrence in all species. The beagle dog is the most satisfactory animal model for studies of the cardiotoxicity produced by anthracyclines. Studies in this species can be conducted using dosage schedules similar to those employed for the treatment of neoplasms in human patients.

Methods of Reducing Anthracycline Cardiac Toxicity

A systematic review was made of all methods which have been employed to prevent or mitigate the chronic cardiotoxicity produced by antineoplastic agents of the anthracycline family. The clinical importance of these agents and the severity of the cardiotoxicity that they can induce has led to numerous attempts to block this untoward effect. These methods were considered in 3 categories: 1. Decreasing myocardial concentrations of anthracyclines and their metabolites. Decreased exposure of the myocardium to the anthracyclines has been accomplished by slowly infusing the drug in order to keep plasma concentrations low or by decreasing the availability of the drug to the myocytes by binding anthracyclines to carrier molecules. 2. Using less cardiotoxic analogues of anthracyclines. 3. Concurrently administering other drugs that will block the cardiotoxic effects of anthracyclines. A variety of different substances have been examined as potential protective agents, including: free radical scavengers, calcium channel blockers, histamine and catecholamine blockers, cardiac glycosides, carnitine and EDTA derivatives (ICRF-159, ICRF-187). ICRF-187 is considered to be the most effective agent currently available for the prevention of anthracycline-induced chronic cardiomyopathy.

Morphological Study of The Cardiotoxicity of Interleukin-2 in Rats

An animal model was developed for the study of the cardiotoxicity produced by the administration of interleukin-2 to patients with metastatic tumors. IL-2 (3 i.p. doses of 5×10^5 Cetus units/day) was given to Sprague-Dawley rats for 2, 3 or 5 days. Cardiac changes consisted mainly of focal lymphocytic and eosinophilic infiltration, myocyte vacuolization, myofibrillar loss and necrosis. Ultrastructural alterations included swelling of endothelial cells, with dissociation of intercellular junctions, migration of lymphocytes into the interstitium, and interstitial hemorrhage and edema. Close contact between infiltrating lymphocytes, particularly large granular lymphocytes, and cardiac myocytes was often observed in areas of tissue damage. Immunoperoxidase stains demonstrated asialo GM₁ ganglioside antibody-positive, granular lymphocytes to be much more frequent in myocardium of IL-2 treated rats than in that of control rats. Our observations suggest that the myocardial damage produced by this agent is triggered by IL-2 activated lymphocytes which exert cytolytic effects, first on endothelial cells and then on cardiac myocytes, thus producing lesions that involve both the cardiac microcirculation and the muscle cells.

Studies on Bioprosthetic Valves

Correlations Between Structure and Function in Porcine Aortic Valve Bioprostheses

To assess correlations between structure and function in porcine aortic valves, a review was made of the anatomic, mechanical and hemodynamic properties pertinent to bioprosthetic valves prepared from porcine aortic valves. The functional effectiveness of the aortic valve is the result of: 1) a dynamic aortic root; 2) the ability of the cusps to close in response to small changes in pressure, and 3) the sharing of mechanical and dynamic stresses between the cusps and the sinuses of Valsalva. Stresses are generated as a consequence of the pressure difference across the closed cusps and reversal of cuspal curvature as the valve opens and closes during the cardiac cycle. Opening and closing bending stresses are present radially, while static loading stresses are distributed circumferentially. The composition and organization of the collagen, elastin and proteoglycans within the aortic valve cusp and its attachment to the sinus of Valsalva minimize the stresses generated within the cusps during the cardiac cycle. The mechanical properties of the aortic valve mirror the structural organization of the connective tissue components within the cusp. The circumferentially oriented collagen bundles within the fibrosa are responsible for maintaining the low circumferential strain in the aortic cusps. The circumferential axis is stronger and stiffer than the radial axis, resulting in a marked anisotropy of cuspal mechanical properties. This anisotropy is the direct consequence of collagen being oriented in a circumferential direction, primarily in the fibrosa. The greater extensibility and creep in the radial direction may be attributed to the radially oriented elastic fibers within the ventricularis and fibrosa. Thus, the present study presents a survey of the close, complex relationships between anatomic, mechanical and functional

properties that exist in porcine aortic valves and provides a basis for the interpretation of data obtained on porcine aortic valves used as cardiac valvular bioprostheses.

Human Aortic Valve Allografts: Structural Changes Preceding Cryopreservation.

To evaluate the occurrence of morphologic changes related to the time delay required for processing human aortic valves to be employed as cardiac valve allografts in heart valve replacement, twenty-five human cryopreserved valves with harvest-related warm ischemic times (WITs) ranging from 0 to 20 hours were studied by light and electron microscopy to characterize the effects of harvesting and preservation on leaflet matrix cells. Each connective tissue cell observed was graded for reversible and irreversible cellular injury. The data obtained were subjected to a Cochran-Mantel-Haenszel trend analysis. The results demonstrated a progression in cellular injury with increasing WIT. During the first 12 hours of warm ischemia, reversible cellular injury predominated. A positive correlation between increasing WIT and reversible cellular injury through the first 12 hours was observed. Minimal morphologic evidence of irreversible injury was noted in valves harvested after less than 12 hours of warm ischemia; however, after 12 hours there was a marked increase in irreversible cellular injury. The data in the present study demonstrate a progression in cellular injury with increasing warm ischemic times. There was virtually no morphologic injury in valves with harvest-related warm ischemic times less than 2 hours and minimal irreversible cellular injury was observed in valves exposed to 12 hours or less of warm ischemia. If cellular viability is critical to homograft durability, then harvest-related warm ischemia may need to be restricted to 12 hours.

Studies on Pulmonary Pathology

Hyperoxia

A study was made to determine whether or not ICRF-187, a chelator of iron, has a protective effect against the acute pulmonary damage produced in rats by exposure to pure oxygen. Histological and ultrastructural studies were made of the lungs of rats that were exposed to 100% oxygen for 60 hours and were treated with either normal saline or with ICRF-187. This compound chelates iron, a metal which is needed to catalyze the formation of toxic oxygen free radicals. Seven of the ten saline-treated rats exposed to oxygen died prior to the end of the study whereas only one of the 10 rats in the ICRF-187 treated group died. All saline-treated rats showed light and electron microscopic evidence of pulmonary damage. ICRF-187 attenuated the morphologic alterations induced by hyperoxia (intraalveolar edema, inflammatory exudates, and bronchiolar epithelial cell swelling and hyperplasia) Capillary thrombi, endothelial cell alterations and alveolar epithelial cell damage also were less severe in ICRF-187-treated rats. ICRF-187 may provide a new and useful approach for the prevention of hyperoxia-induced pulmonary damage.

Annual Report of the Section on Pulmonary and Molecular Immunology
National Heart, Lung, and Blood Institute
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In the past year, the research of the Section on Pulmonary and Molecular Immunology has centered on the study of select proteins critical to the process of T-cell activation. T-cells play a central role in mounting an immune response and as such are intimately related to components of each of the three principal disease area focuses of NHLBI. This report summarizes studies related to (1) IL-2 receptor structure and function (2) Molecular regulation of IL-2 receptor α , β , and γ chain genes and related work on NF- κ B, and (3) The Act-2 cytokine. The IL-2 receptor has been a longstanding focus of this group, members of which were the first to discover the existence of both the IL-2R α and IL-2R β chains and to clone cDNAs encoding the IL-2R α chain. The group has continued its high productivity in this area with the major finding this year that mutation of the IL-2R γ chain results in X-linked severe combined immunodeficiency (XSCID) in humans, a finding with major clinical and basic research implications, as detailed below. Research also relates to other components of structure, function, and molecular regulation of the IL-2R α , IL-2R β , and IL-2R γ chain genes. Because of the critical role of the NF- κ B binding site in the IL-2R α chain gene in mediating induction in response to HTLV-I infection, specific aspects of this transcription factor have been studied. In addition, the group has focused some effort on a chemotactic cytokine, denoted Act-2, that it was the first to discover in 1988. Each of these areas will be discussed in turn.

IL-2 receptor- structure and function and the discovery that IL-2R γ mutation results in XSCID.

The human interleukin-2 receptor is being studied to understand critical components of the T cell immune response in normal and neoplastic cells. When T-cells are activated by antigen or mitogenic lectin, both IL-2 and IL-2 receptor expression are induced. IL-2 and IL-2 receptors control the magnitude and duration of the T-cell immune response based on the amount of IL-2 produced, the levels of receptors expressed, and the time course of each of these events. Whereas low levels of intermediate affinity IL-2 receptors are expressed on resting cells, following antigen stimulation, expression of both high and low affinity IL-2 receptors is potently induced. At least three chains of the IL-2 receptor are now known to exist, namely IL-2R α , IL-2R β , and IL-2R γ . Other proteins may additionally contribute to a multichain receptor complex. In the past year, major advances have been made in a number of areas.

The most significant finding of the year was that mutation of IL-2R γ results in X-linked severe combined immunodeficiency (XSCID) in humans. This observation resulted from the group's observation (based on analysis of rodent-human somatic cell hybrids and by fluorescent in situ hybridization) that the IL-2R γ gene is located on the X chromosome in a position (Xq13) previously determined to be the location for XSCID. This analysis was refined by using single stranded conformational polymorphism (SSCP) to identify polymorphisms within introns 1 and 2 which allowed a genetic linkage analysis. This analysis indicated that IL-2R γ was tightly linked to the XSCID locus. Finally, DNA was prepared from EBV transformed B cell lines derived from three XSCID patients. DNA sequencing was performed by nested PCR amplification of genomic DNA followed by DNA sequencing on magnetic beads. Each of the three unrelated XSCID patients was found to have a different point mutation, resulting in a different premature stop codon. Thus, mutation of the IL-2R γ gene results in XSCID. Since XSCID is characterized by an absence of or significantly diminished numbers of T cells, these data indicate that IL-2R γ is required for thymic

maturation of T lymphocytes. Interestingly, IL-2 deficient mice and human SCID patients deficient in IL-2 production have normal levels of T cells. These data together suggest that XSCID and IL-2 deficiency differ in the degree of maturation of T cells. Thus, it is reasonable to speculate that IL-2R γ is a component of more than one cytokine receptor system, an hypothesis the group has confirmed. This is an area of active investigation in the lab. A project to perform gene therapy for XSCID has been initiated.

To facilitate the preparation of a murine model, the murine IL-2R γ cDNA has been cloned and sequenced, and the murine IL-2R γ gene has been identified and sequenced. These reagents are being used to facilitate the preparation of a construct to generate an IL-2R γ "knockout" mouse by homologous recombination. IL-2R γ expression is also being studied in murine thymocyte populations to help elucidate the role it plays in T-cell development.

The lab has made significant progress in studies utilizing the expression of the IL-2R α and β chain cDNAs in 32D myeloid progenitor cells to elucidate features of IL-2 mediated signal transduction. Such cells were prepared previously in the lab. 32D cells are normally dependent on IL-3, but when transfected with IL-2R β , they also respond to IL-2. 32D- β cells are therefore valuable for studying IL-2 signal transduction since these cells respond to IL-2 but do not require maintenance in IL-2 for survival. Inhibitors of tyrosine kinases such as herbimycin A inhibit both IL-2 induced proliferation and IL-2 induced IL-2R α mRNA expression in 32D- β cells, indicating that tyrosine kinase(s) are required for IL-2 induced signaling. The group has found that tyrosine kinase inhibitors not only are capable of inhibiting proliferation in these cells but can also regulate apoptosis and *bcl-2* expression in these growth factor dependent cells. Specifically, either IL-2 or IL-3 can upregulate *bcl-2* mRNA levels; this upregulation is blocked by herbimycin A. Transfection of a *bcl-2* expression vector not only prolongs survival following growth factor withdrawal but also confers resistance to herbimycin A. Thus, PTKs are involved in the regulation of apoptosis and *bcl-2* expression, but herbimycin sensitive PTKs appear not to be required for the action of Bcl-2 since Bcl-2 can exert its growth survival effect even when these PTKs are inactivated. Multiple IL-2R β mutants and IL-2 receptor chimeric constructs have been prepared and are being utilized in transfection experiments to further study the mechanisms of IL-2 signaling.

4. Identification of functional IL-2 receptors on polymorphonuclear neutrophils (PMNs). We have reported that PMNs express IL-2R β mRNA and protein and can respond to IL-2. These cells express typical intermediate affinity IL-2 receptors, binding IL-2 with a K_d of 1.1 nM. Not only are receptors present, but IL-2 can induce augmentation of TNF- α production, with increased cytotoxicity to tumor target cells. The release of TNF- α was inhibited by an antibody against human IL-2R β . Maximal TNF- α mRNA was detected 3 h after IL-2 exposure, followed by continuous maintenance of high mRNA levels for 18 h. Maximal TNF- α transcription was found at 1000 U/ml IL-2, which achieved levels comparable to those found with GM-CSF, IL-8, and *Candida albicans*. Using actinomycin D, it is clear that new and continuous synthesis of a labile TNF- α mRNA was responsible for the observed high levels of transcripts. Cycloheximide did not affect TNF- α mRNA induction in IL-2 treated PMNs, but abrogated it in GM-CSF treated PMNs and superinduced it in *C. albicans*-treated PMNs. Thus, various control elements must be involved in the transcription of the TNF- α gene. The induction of TNF- α and functional activation of PMNs by IL-2 is therefore an important immunomodulatory property of IL-2 that was not previously recognized. It was further noted that PMNs were able to respond functionally to IL-2 by enhanced growth inhibitory activity against *C. albicans*, an opportunistic fungal pathogen. Activation of antifungal activity was dose dependent, with

significant elevation of function detected at 1U/ml of IL-2, achieving maximal activation at 1000 U/ml. The action of IL-2 was rapid, with maximal PMN activation after 30 minutes of IL-2 treatment. The IL-2 enhancement could be blocked by an antibody to IL-2R β , but not by an antibody to IL-2R α . Analysis of the mechanism of IL-2 activation revealed that oxidative metabolism, as measured by superoxide anion production, was not involved. Instead, release of lactoferrin by PMNs appeared to be responsible for the enhanced killing of *C. albicans*. Not only was lactoferrin detected in the supernatants of IL-2 treated PMN, but also the antifungal activity of PMN activated by IL-2 could be blocked in the presence of anti-lactoferrin. These reports represent the first demonstration of IL-2 receptors on neutrophils and represents an important elucidation for a role for IL-2 in a non-lymphoid cell.

Interleukin-2 receptor – molecular regulation

IL-2R α chain gene regulation: The IL-2R α chain 5' regulatory sequences were previously delineated by our group, with characterization of an enhancer element which spans an NF- κ B site, a CArG motif, an Sp1 site, and a fourth site denoted NF-IL-2RA, all located between -299 and -228 relative to the major transcription initiation sites. We have studied the significance of other elements further downstream of the previously described enhancer, and a second enhancer element has been identified. This element contains binding sites for two transcription factors– an Ets family protein and HMG-I/Y. The Ets family protein, whose specific identity has been established, is critical for both IL-2R α enhancer and promoter activity. Investigations are focused on whether an accessory protein is required for optimal binding of this factor to the IL-2R α 5' regulatory region. A binding site selection analysis is being performed to elucidate optimal binding sites for this factor. A large number of enhancer and promoter constructs are being evaluated to further elucidate the role played by these proteins. Identification of the new enhancer and associated transcription factors may represent critical discoveries which may help to explain the inducibility of the IL-2R α gene even in a setting in which NF- κ B cannot contribute to IL-2R α gene activation.

In parallel, we have pursued experiments using in vivo footprinting methodology. These experiments were motivated by the observation that there appear to be at least two different molecular mechanisms for activating the IL-2R α gene. Specifically, mutation of the κ B site results in a loss of IL-2R α -CAT activity in HTLV-I infected cells but not in Jurkat cells, whereas mutation of the CArG motif (which can bind serum response factor in vitro) appears to be more important for IL-2R α -CAT activity in PMA stimulated Jurkat cells than in HTLV-I transformed cells. Since there is no PMA-induced binding to the CArG motif in vitro, we are investigating whether changes in binding might be seen in vivo. Preliminary results suggest that in vivo, PMA does induce binding to the CArG motif. We hope that better correlation of functional results will be found with in vivo binding analyses than has been found previously with in vitro binding studies.

IL-2R β chain gene regulation: The 5'-flanking region of the human IL-2R β gene was previously reported by the group to possess promoter activity. Nested 5' deletion and internal deletion mutants have now been used to delineate cis-acting elements critical for basal and PMA-inducible IL-2R β promoter activity. The region downstream of -363 is critical for both basal and PMA inducible expression and contains at least three enhancer-like regions. Among them, the -56 to -34 enhancer was the most potent and had high level activity in two T cell lines but not in HeLaS3 cells. This enhancer contains a GGAA Ets binding site. Ets family members encode transcription factors that

interact in a sequence-specific manner with purine-rich motifs in promoters and enhancers of viral and cellular genes encoding important immunological regulatory proteins, including the polyomavirus enhancer, the moloney sarcoma virus (MSV) long terminal repeat (LTR), the human T-cell receptor (TCR) α -chain enhancer, the IL-2 enhancer and the class II MHC promoter. The IL-2R β Ets site binds Ets-1 and GABP *in vitro*, two different Ets family proteins. This conclusion is based on experiments using electrophoretic mobility shift assays, DNaseI footprinting, and methylation interference analyses. Mutation of the Ets motif strongly diminished both promoter and enhancer activities. Thus, this site plays a key role in regulating basal and PMA-inducible IL-2R β activity and may also contribute to tissue specific expression of the IL-2R β gene. We have also begun to identify proteins binding to the other enhancers. In one of these, a PMA inducible binding activity has been found which appears distinct from AP-1 and NF- κ B. The factor(s) responsible for this binding activity are being characterized. Since IL-2R β mRNA is potently induced in normal human peripheral blood lymphocytes by the protein kinase C activator PMA, the T cell mitogen PHA and anti-CD3 antibodies, and somewhat less strongly induced by IL-2, we hypothesize that the inducible enhancers we have identified may play a role in these physiological modes of induction of the IL-2R β gene.

IL-2R γ gene regulation. The IL-2R γ gene was isolated and characterized. It is composed of eight exons and seven introns and spans approximately 4.2 kb. Analogous to the IL-2R β gene, the two pairs of conserved cysteines typical of cytokine receptor superfamily proteins are located in adjacent exons, and the conserved WSXWS motif is located in the exon preceding the one that encodes the transmembrane domain and a small part of the cytoplasmic domain. In each gene, the remainder of the cytoplasmic domain is encoded by the final two exons. Cross-hybridizing sequences were found in other mammalian species examined but not in yeast. Three principal transcription initiation sites were mapped by a combination of primer extension and ribonuclease protection assays. The region 5' to the transcription initiation sites exhibited promoter activity when cloned upstream of the luciferase reporter gene. The IL-2R γ gene was mapped to the X chromosome at Xq13. As discussed above, based on linkage analysis and DNA sequencing, we determined that mutation of the IL-2R γ gene results in XSCID in humans. Based on Northern blot analysis, in contrast to IL-2R α and IL-2R β , IL-2R γ mRNA levels are not significantly induced following mitogenic stimulation of peripheral blood lymphocytes.

Oxidation-reduction and NF- κ B binding. The IL-2R α gene can be activated by ADF (adult T cell leukemia derived factor), a protein produced by HTLV-I transformed T cells and which has been demonstrated to be homologous to thioredoxin. Given the importance of the NF- κ B binding site for IL-2R α induction in HTLV-I transformed cells, it is provocative that the group previously found that NF- κ B binding *in vitro* is abrogated by oxidation or alkylation, suggesting that the redox state may be involved in regulating HTLV-I induced IL-2R α expression and NF- κ B binding. This year, the group reported that of the 7 cysteines, only Cys 62 mediates the *in vitro* sensitivity to oxidation and alkylation. It was further reported that Cys 62 is located within a region critical for p50 binding but not dimerization and that certain point mutations of p50 in this region function as transdominant negative mutants. It was also reported that the residues important for binding are highly conserved in all NF- κ B family proteins, including p65, the c-Rel oncoprotein, and *Drosophila dorsal* maternal effect gene, and it was demonstrated that the same region of p65 is involved in DNA binding. Finally, it was reported that limited N-terminal regions of p50 and p65 appear to significantly contribute towards the differences in binding specificity manifested by p50 and p65. Analysis of this DNA binding domain allowed us to define a highly specific motif R[F/G][R/K]YXCE present not only in

all NF- κ B family proteins but also in only those zinc finger proteins which are capable of binding to κ B sites. Preliminary mutagenesis experiments in α -CRYBP1, a double zinc finger protein which binds to κ B sites, indicate that the motif contains residues critical for DNA binding of this protein, although it unknown whether the role of the residues in these zinc finger proteins is identical to that played in the binding of NF- κ B family proteins

Studies related to the Act-2 cytokine:

In 1988, the group discovered Act-2 as a gene which was expressed in activated but not resting T cells. It was found to encode a secreted protein of 69 amino acids in length. Act-2 is a member of a family of small secreted proteins, many members of which have inflammatory or chemotactic activities. This family, whose members are now denoted as "chemokines" can be divided into two subfamilies based on whether the first two of four conserved cysteines are adjacent (CC) or separated by one amino acid (CXC). Act-2 is a member of the CC subfamily, and is presumed to be the human homologue of murine macrophage inflammatory protein (MIP)-1 β . Synthesis and secretion of this cytokine are rapidly induced in T cells, B cells, and monocytes following stimulation with antigen or mitogen. Act-2 has recently been demonstrated by others to be specifically chemotactic for CD4+ T lymphocytes. During the past year, we reported that Act-2 protein has activity as a bone marrow stem cell inhibitor and can efficiently compete with murine MIP-1 α for binding. In addition, Act-2 protein was produced in both bacterial and baculovirus expression systems and structural analysis of the protein is underway. After confirming quality of the recombinant Act-2 protein by one dimensional NMR, more than 10 mg each of unlabeled, 15 N-labeled, and 15 N plus 13 C doubly labeled Act-2 protein were produced by multidimensional NMR. The structural analysis is in progress. Act-2 is also being made to prepare crystals for Xray crystallography. Structural information is vital to eventual drug agonist/antagonist development as well as being scientifically important since no structural information is yet available on any members of the CC subfamily.

Annual Report of the Pulmonary Branch
National Heart, Lung, and Blood Institute
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In the past year, the Pulmonary Branch basic and clinical research program has been focused in two areas: I. Gene therapy, including in vivo gene transfer strategies for the lung and other organs; and II. Lung inflammation relevant to the pathogenesis and therapy of acute and chronic lung disease.

I. Gene Therapy

CF is an autosomal recessive disorder resulting from mutations of the cystic fibrosis transmembrane regulator (CFTR) gene, a 27 exon gene occupying 250 kb of chromosome 7. All organs with exocrine glands are affected, but the major clinical manifestations are in the lung, with impacted mucus, chronic infection, inflammation, and airway and parenchymal lung derangements. Studies in the past year regarding gene therapy for cystic fibrosis have focused on both the respiratory and gastrointestinal manifestations of the disease.

A strategy for somatic gene therapy of CF is direct transfer of the normal CFTR gene to CF airway epithelial cells using a replication deficient recombinant adenovirus (Ad) vector. The feasibility of this approach has been demonstrated in cotton rats (Cell 1992;68:143). However, because Ad DNA is unlikely to integrate in the host cell genome, repetitive administration of the vector may be necessary for this therapy to be effective. One possible hurdle to repetitive therapy may be the development and persistence of an immune response to the vector. To evaluate this possibility, a recombinant Ad vector containing the normal human CFTR cDNA was administered to the airways of cotton rats (10^9 pfu/animal) and serum anti-Ad antibody titers were evaluated by ELISA. By day 7, anti-Ad antibodies were detected above controls in animals receiving intratracheal Ad vector (controls mean 190, range <40-2,560, n=13; Ad vector mean 11,130, range 2,560-40,960, n=9; $p < 0.001$). The anti-Ad antibodies were detectable for at least 4 months (controls mean 640, range 640-640, n=3; Ad vector mean 98,100, range 40,960-327,680, n=4; $p < 0.001$). The feasibility of repetitive administration in the presence of the anti-Ad antibody response was then evaluated using two different Ad vectors [Ad- α 1AT, containing the human α 1-antitrypsin cDNA, and Ad.RSV- β gal, containing the E. coli lacZ (β -galactosidase) gene]. Ad- α 1AT was administered intratracheally and followed 99-106 days later by intratracheal Ad.RSV β gal. After 24 hr, animals were evaluated for serum antibodies and presence of lung β -galactosidase (β -gal) activity. Importantly, despite the presence of high serum anti-Ad antibody titers, β -gal activity was easily detected in epithelial cells of the airways. These observations demonstrate that replication deficient recombinant Ad vectors generate persistent serum antibodies in cotton rats, but that even in the presence of serum antibodies, successful respiratory epithelium gene transfer and expression can still be accomplished.

Cystic fibrosis (CF) results from mutations of the CFTR gene and consequent defective regulation of cAMP-mediated Cl^- permeability across epithelial cell apical membranes. Given that in vitro transfer of normal CFTR cDNA corrects the defect and that the recombinant adenovirus (Ad), AdCFTR,

transfers the human CFTR cDNA in vivo to the airway epithelium of experimental animals, CF may be amenable to Ad-mediated gene therapy. One concern is whether the degree of CFTR overexpression achieved by AdCFTR may have untoward effects on basal and/or cAMP-stimulated Cl^- efflux. To address this, the CF pancreatic epithelial cell line, CFPAC-1, was infected (64 hr) with increasing doses of AdCFTR [0-1000 plaque forming units (pfu)/cell] and analysed for extent of CFTR protein expression [by scoring CFTR positive cells (by immunohistochemical staining)] and function [by evaluating basal and cAMP-stimulated (with forskolin) $^{36}\text{Cl}^-$ efflux rates]. As AdCFTR dose increased: (1) CFTR protein increased proportionally ($r^2=0.93$); (2) basal Cl^- efflux rate did not change ($p>0.05$, all doses); and (3) forskolin-stimulated Cl^- efflux rate initially increased (20 pfu/cell, $p<0.05$ compared to basal), and then stabilized (>20 to 1000 pfu/cell, all $p<0.05$ compared to basal, $p>0.1$ between doses ≥ 20 pfu/cell). Thus, while relatively low levels of normal CFTR reestablish cAMP-stimulated Cl^- efflux in CF epithelial cells, additional CFTR has no additive effect. The dissociation of the amount of CFTR and its function at high CFTR protein levels suggests that cells limit CFTR function and that CFTR overexpression during in vivo gene therapy will have no untoward effects.

All available evidence suggests that if the normal cystic fibrosis transmembrane conductance regulator (CFTR) cDNA could be delivered to all major categories of airway epithelial cells, the respiratory manifestations of CF could be corrected. Studies from our laboratory have demonstrated that this can be readily achieved for the airway surface epithelium using replication deficient, recombinant adenovirus vectors administered via the airways. It is unclear, however, whether the air route can be used to deliver genes to the epithelium of the submucosal glands, as their architecture and mucus filled lumen may provide a difficult barrier. As an alternative approach, based on the knowledge that the pulmonary and bronchial circulations have extensive anastomoses, we hypothesized that gene transfer to submucosal gland epithelium might be achieved if the adenovirus vectors were administered to the isolated pulmonary circulation for 15 min, sufficient time for the virus to reach the bronchial circulation and the submucosal glands. To evaluate this concept, Ad.RSV β gal, a replication deficient, recombinant adenovirus containing the *E. coli* lacZ reporter gene coding for β -galactosidase (β -gal) was injected into the right upper lobe (RUL) branch of the pulmonary artery of sheep, following occlusions of the RUL artery and vein. After 15 min, the pulmonary circulation was restored. Lungs were evaluated 1 to 3 d later by histochemical analysis for β -gal activity. In contrast with uninfected lobes, β -gal activity was detected in the right upper lobe, with β -gal activity in the endothelium of the pulmonary and the bronchial circulation, including arteries, veins, and capillaries. Airway epithelial cells in bronchi and in bronchioles expressed β -gal. Importantly, the epithelium of the submucosal glands (primarily composed of serous cells in sheep) also expressed β -gal in either a patchy or diffuse distribution, depending on the location. These observations suggest that the anastomoses between pulmonary and bronchial circulation may enable targeting of all categories of cells necessary for gene therapy for CF using an adenovirus vector.

Lung disease associated with disorders such as cystic fibrosis (CF) may be amenable to somatic gene therapy in which there is delivery of the normal gene directly to the respiratory epithelium. Adenovirus (Ad) vectors are

particularly attractive for gene transfer to the lung for they are tropic for the respiratory epithelium, do not require target cell replication for gene expression, and are capable of transferring genes to the respiratory epithelium in vivo (Cell 1992;68:143-155). For safety reasons, Ad vectors can be rendered replication deficient by deletion of the early region 1a (Ela). One concern, however, is the possibility of the Ela⁻ replication deficient vector evolving into a replication competent virus through recombination with part of the Ad genome (containing Ela) present in the respiratory epithelium in a partial form or as latent Ad. To assess the extent of this risk, we evaluated respiratory epithelial cells obtained from normals (n=91) and individuals with CF (n=43) for the presence of Ela sequences. Respiratory epithelial cells were obtained from the inferior turbinates of the nose (by direct visualization) and from trachea and/or main bronchi by bronchoscopy, using a standard cytology brush. Cell number was determined by quantifying the number of Alu sequences in the DNA of an aliquot of the cell lysate compared to a standard curve of highly purified human genomic DNA. To detect Ela sequences, the DNA in an aliquot of cell lysate was amplified by polymerase chain reaction (PCR), using Ela specific primers and a "nested" Ela probe compared to a standard curve of Ad type 2 DNA, with a sensitivity of ≥ 4 Ela copies/ 10^3 cells. Ela sequence was detected in the respiratory epithelium of 19 of 91 normals (21%), with the Ela copy number in the positive samples $55 \pm 18 / 10^3$ cells. In CF individuals, 5 of 43 (12%) had detectable Ela sequences, with the Ela copy number in the positive samples $104 \pm 22 / 10^3$ cells. These results demonstrate the presence of Ad Ela sequences in the respiratory epithelium of a small percentage of individuals, and therefore suggest the need for assessing for the presence of Ela in potential candidates for Ad-Ela⁻-vector mediated pulmonary gene therapy.

Exocrine pancreatic dysfunction is common in cystic fibrosis, with fat and protein malabsorption a chronic, debilitating clinical problem. As an initial approach to developing a gene therapy strategy for pancreatic enzyme replacement in cystic fibrosis, we evaluated the feasibility of using an Ela⁻ replication deficient recombinant adenovirus to transfer the cDNA for human pancreatic lipase, a 48 kDa pancreatic enzyme that catalyzes the intraduodenal conversion of triglycerides into fatty acids and monoglycerides. To accomplish this, the adenovirus vector AdCMV.lip was constructed using the cytomegalovirus immediate early promoter to drive the 1.5 kb human pancreatic lipase cDNA. In vitro infection of COS-7 cells with AdCMV.lip (10^8 pfu/ 10^6 cells) resulted in the production of 7.6 ± 4.1 units active lipase/24 hr- 10^6 cells. Since the exocrine pancreas is damaged early in cystic fibrosis, and thus is an unlikely site for successful pancreatic enzyme gene transfer, the gall bladder was evaluated as a potential alternative in vivo target. To evaluate the feasibility of this approach, 2×10^{10} pfu AdCMV.lip was instilled ex vivo into the lumen of sheep gall bladder. Following 24 hr at 37°, analysis of the fluid in the gall bladder lumen demonstrated 428 ± 69 milliunits active lipase/ml, a level significantly greater than control virus infected gall bladder ($p < 0.05$). These observations demonstrate that an adenovirus vector can be used to transfer a pancreatic enzyme in vitro and ex vivo, suggesting the feasibility of in vivo gene therapy strategy for exocrine pancreas enzyme deficiency states such as that in cystic fibrosis.

One of the important elements in designing gene therapy for the respiratory

manifestations of CF is the choice of the promoter to control the CFTR cDNA to be transferred. One promoter that may serve this purpose is that for secretory leukoprotease inhibitor (SLPI), a serine antiprotease, that helps to protect airway epithelial surface from the destructive capacity of neutrophil elastase. Studies of biological fluids and immunohistochemical evaluation of tissue suggest that the SLPI gene is expressed in a tissue specific manner at mucosal surfaces, including those of the lung, cervix, parotid duct, and seminal vesicles. To evaluate molecular mechanisms involved in the mucosal specific expression of the SLPI gene, we used cell types that expressed the gene to evaluate the promoter function of sequences 5' to exon I. SLPI mRNA transcripts were expressed in human epithelial cell lines of mucosal origin (lung-derived HS-24 and cervix-derived HeLa), but not in fibroblasts HFL-1 or an erythroleukemia cell line K562. Consistent with the mRNA data, transfection of the 1.2 kb 5'-flanking region linked to a luciferase reporter gene demonstrated active promoter function in HS-24 (9% of the Rous sarcoma virus long terminal repeat as a control promoter) and HeLa (16%), but little activity in HFL-1 (1%) or K562 (<1%). To evaluate possible cis-acting DNA sequences important for transcriptional regulation of SLPI expression, sequentially deleted 5'-flanking regions of the SLPI gene linked to a luciferase reporter gene were evaluated in HS-24 cells. This analysis demonstrated that cis-acting sequences spanning nucleotides -115 to +16 relative to the transcription start site gave equivalent promoter function to increasing lengths of 5'-flanking region up to -1228 to +16 i.e., a short length of 5'-flanking region is effective for constitutive promotion of the SLPI gene. Interestingly, this short 5' fragment (-115 to +16) was markedly upregulated by phorbol esters, with promotion of luciferase activity in HS-24 cells in the presence of 100 nM PMA increasing to 60% that of the control RSV promoter. These data suggest that the SLPI promoter is constitutively driving the SLPI gene at relatively high levels and that it can be regulated at the transcriptional level by inflammatory stimuli. In the context that SLPI gene expression is limited to specific epithelial cell types, these 5' elements may be useful as regulatory elements for mucosal specific gene therapy.

Expression of the cystic fibrosis transmembrane conductance regulator (CFTR) gene, a constitutive "housekeeping-type" gene, can be down-regulated at the transcriptional level by inflammatory stimuli as demonstrated in vitro with phorbol myristate acetate (PMA) as a model. The ability of inflammatory stimuli to down-regulate CFTR gene expression is a potential problem for gene therapy for the respiratory manifestations of cystic fibrosis (CF), since the established inflammatory milieu in individuals with CF may down-regulate expression of the newly transferred gene, thus prohibiting correction of the basic abnormalities in the airway epithelium. To evaluate designs of promoter-CFTR cDNA that could be transferred to human epithelial cells but would be resistant to this form of down-regulation, two different promoter-CFTR cDNA constructs [5' long terminal repeat (LTR) of the Moloney murine leukemia virus-normal human CFTR cDNA; major late promoter (MLP) of adenovirus (Ad) type 2-normal human CFTR cDNA] were transferred to T84 human colon carcinoma cell line (normally expressing the CFTR gene and cAMP-mediated Cl⁻ secretory function) via a retroviral or adenoviral vector, respectively. Retrovirus-modified monoclonal T84 cells expressing the endogenous (6.5 kb) and exogenous (8.3 kb) CFTR mRNAs were selected with neomycin. Exposure of parental T84 cells to PMA (100 nM, 24 hr) down-regulated endogenous CFTR transcript levels and, in parallel,

reduced cAMP-stimulated Cl^- secretion. In contrast, cells modified with the retrovirus LTR promoter-CFTR cDNA unit still maintained normal CFTR function, i.e., cAMP-regulated Cl^- secretion, with the persistence of the exogenous CFTR transcripts even after exposure to PMA. Likewise, cells evaluated 48 hr after modification with the Ad MLP-CFTR cDNA unit maintained normal CFTR function in the presence of PMA. These in vitro data suggest that constitutive viral promoters will be resistant to inflammation-induced down-regulation, a useful feature for design of vectors to be used for correction of CF by gene transfer.

Another important element in strategizing gene therapy for CF is to identify target cells that may be progenitor cells for the airway epithelium. Basal cells are small, ovoid or pyramidal shaped, basement membrane-attached cells most prevalent in humans in the proximal airways. Studies in experimental animals suggest that basal cells can serve as precursors of other airway epithelial cells. To evaluate this concept in humans, we hypothesized that if basal cells are capable of differentiating into other airway epithelial cell types, gene expression of cell-specific proteins might begin in basal cells, providing evidence of a precursor-product relationship. To accomplish this, we examined the localization of mRNA transcripts and protein products of the secretory leukocyte protease inhibitor (SLPI) and Clara Cell 10 kDa protein (CC10) genes in human proximal airway epithelium obtained by biopsy (n=5) and epithelial cells obtained by brushing (n=12) airways of normals at bronchoscopy. Cell types were characterized by morphological appearance, cytochemical staining (PAS) and the use of cytokeratin 14 (basal cell-specific) and 18 (differentiated cell-specific) antibodies. mRNA transcripts were localized by in situ hybridization, and protein localization was achieved by immuno-histochemistry. In situ hybridization with probes specific for CC10 and SLPI mRNA transcripts demonstrated transcripts for CC10 expressed predominantly in basal cells, with lesser amounts in some non-ciliated columnar and secretory cells. Likewise, SLPI mRNA transcripts were abundant in basal cells of the surface epithelium, as well as in serous cells within the submucosal glands. As a control, γ -actin mRNA transcripts were present in all cell types. In contrast, CC10 protein was present in non-ciliated columnar cells and some mucous cells, while SLPI protein was found only in serous cells. Neither CC10 or SLPI protein were present in basal cells. Thus, while there is abundant mRNA expression in basal cells for both the CC10 and SLPI genes, CC10 protein expression appears to be restricted to non-ciliated columnar and mucous cells, and SLPI protein expression to serous cells. This strongly suggests that basal cells can serve as precursor cells for some other populations of epithelial cells in the human proximal airway epithelium.

In addition to CF, the Pulmonary Branch has focused efforts toward gene therapy of CNS disorders, hematologic disorders, α_1 -antitrypsin deficiency, oxidant-damage of the airway epithelium, and lung cancer.

A variety of disorders of the central nervous system (CNS) can potentially benefit from local delivery of genes coding for therapeutic proteins or peptides. To evaluate the strategy of using El^- replication deficient recombinant adenovirus (Ad) to directly transfer genes to CNS, the Ad vectors Ad.RSV β gal [containing the intracellular marker gene β -galactosidase (β gal)] and Ad- α 1AT [containing the human α 1-antitrypsin (α 1AT) cDNA, a model secreted protein] were administered directly to rats via a cannula in

a lateral ventricle to target ependymal cells (and thus access of a secreted gene product to both the cerebral spinal fluid and the brain parenchyma) or via stereotactic injection in the region of the globus pallidus and striatum (to demonstrate direct transfer to specific parenchymal areas). Whereas control animals showed no histologic evidence of β gal activity in the CNS, when Ad.RSV β gal was administered in the lateral ventricle, rats evaluated 4 d later had striking β gal expression in ependymal cells of the cerebral ventricles, as well as in the leptomeningeal layer on the brain surface. Following administration of Ad- α 1AT into a lateral ventricle, analysis of CSF demonstrated dose-dependent levels of human α 1AT 2 to 6 d later. When the adenovirus vector Ad.RSV β gal was administered directly to the globus pallidus and striatum of experimental animals, β gal activity was detected at the site of administration as well as in the ipsilateral substantia nigra. These observations demonstrate that Ad vectors are capable of direct in vivo delivery of genes to the CNS, suggesting a variety of possibilities for future gene therapy applications.

Erythropoietin (EPO), a 34 kDa glycoprotein, is the principal regulator of erythropoiesis. To evaluate the concept that in vivo gene transfer might be used as an alternative to recombinant human EPO in applications requiring 1 to 3 wk stimulation of erythropoiesis, the replication deficient recombinant adenovirus (Ad) AdMLP.Epo was constructed by deleting the majority of E1 from Ad type 5, and replacing E1 with an expression cassette containing the Ad type 5 major late promoter (MLP) and the human EPO gene, including the 3' cis-acting hypoxia response element. In vitro studies demonstrated that AdMLP.Epo infection of COS-7 cells resulted in the production of 17 ± 2 units of human EPO/24 hr- 10^6 cells. Further, infection of the human hepatocyte cell line Hep3B with AdMLP.Epo resulted in a 15-fold increase in EPO production in 24 hr that was enhanced to 116-fold in the presence of a hypoxic stimulus. One time in vivo administration of AdMLP.Epo (7×10^9 pfu/kg) to the peritoneum of cotton rats caused a marked increase in red cell production, with a 2.6-fold increase in bone marrow erythroid precursors by day 4, and a 7-fold increase in reticulocyte count by day 7. The hematocrit increased gradually from the baseline ($46 \pm 2\%$) to a maximum of $64 \pm 4\%$ at day 14, and $55 \pm 1\%$ at day 24. This study demonstrates a striking physiologic response to in vivo gene transfer, and suggests that gene therapy with a single administration of an Ad vector containing the human EPO gene may provide an alternative means of significantly augmenting the circulating red cell mass over the 1 to 3 wk period necessary for many clinical applications.

In vivo gene therapy for α 1-antitrypsin (α 1AT) deficiency offers the potential of augmentation of α 1AT levels by modifying cells of the affected individual to produce α 1AT. The peritoneal mesothelium is potentially an excellent target for such modification in that it offers the advantages of easy access and an extensive surface area that can readily exchange macromolecules with the circulation. To evaluate the mesothelium as a potential site for in vivo gene transfer, Ad.RSV- β gal, a replication deficient recombinant adenovirus (Ad) vector containing the E. coli lacZ (β -galactosidase) gene driven by the Rous sarcoma virus long terminal repeat was administered to the peritoneum of cotton rats [10^9 plaque forming units (pfu)]. Histochemical staining after 24 hr demonstrated β -galactosidase activity in the peritoneal mesothelial cells. On this basis, Ad- α 1AT, an Ad vector containing the human α 1AT cDNA driven by the adenovirus type 2 major

late promoter was administered in a similar fashion into the peritoneal cavity of cotton rats. Human α LAT was detected in serum at doses of 10^8 pfu/animal, with maximum values at $\geq 10^9$ pfu/animal. Human α LAT was detectable in serum for up to 24 days with highest levels of $2.3 \pm 0.4 \mu\text{g/ml}$ at day 4. Polymerase chain reaction analyses documented adenovirus driven human α LAT mRNA transcripts in peritoneal mesothelial cells but not in other tissues evaluated (lung, heart, liver, spleen, kidney, brain and testes), confirming the mesothelium as the site of production of the human α LAT. After administration of the Ad vectors, serum antibodies to adenoviruses were detected, with titers up to 10^5 . Despite this, repeat intraperitoneal administration of Ad- α LAT, 3 months after an initial Ad- α LAT administration, yielded detectable levels of human α LAT in serum. These data demonstrate the feasibility of adenovirus-mediated gene transfer targeted to the peritoneal mesothelium to synthesize and secrete human α LAT in vivo, and suggest that the peritoneal mesothelium may be a suitable target for in vivo gene therapy for α LAT deficiency.

A variety of lung disorders are characterized by oxidant-mediated injury to the airway epithelium. Based on the knowledge that H_2O_2 plays a central role in oxidant-mediated airway epithelial injury, and that the airway epithelium has minimal defenses against H_2O_2 , we designed a replication deficient recombinant adenovirus containing the human catalase cDNA (AdCL) to transfer the catalase cDNA to bronchial epithelial cells to augment intracellular anti- H_2O_2 protection. When infected in vitro with AdCL, human bronchial epithelial cells obtained by cytologic brush expressed AdCL-driven catalase mRNA detected by northern analysis, and had a 3 to 8-fold increase in catalase activity compared to controls 1 to 7 days after infection. To evaluate the antioxidant protection resulting from AdCL infection, IB3-1 cells, a human bronchial epithelial cell line from a cystic fibrosis patient, were infected in vitro with AdCL or a control adenovirus. Three days later, when exposed in vitro to lethal doses of H_2O_2 (2 mM) for 1 day, the majority of AdCL infected cells survived, compared to controls which were significantly damaged or dead. Importantly, when AdCL was administered in vivo into cotton rat airways, high levels of AdCL-driven catalase mRNA were detected by northern analysis. Thus, a recombinant adenovirus containing the human catalase cDNA is able to infect bronchial epithelial cells and to express high levels of catalase to protect cells in vitro against H_2O_2 -mediated oxidant stress. This, together with the demonstrated ability to transfer the catalase cDNA to the lungs in vivo, supports the feasibility of adenovirus-mediated transfer of catalase cDNA to human bronchial epithelium to protect against oxidant injury.

Tumor necrosis factor- α (TNF), a potent cytokine with anti-tumor activity, has limited therapeutic utility for lung cancer due to relative insensitivity of the tumors and toxicity of TNF. Based on the hypothesis that production of TNF in the local tumor milieu may evoke an in vivo response to suppress tumor growth, the human TNF cDNA was transferred to human lung cancer cell lines (A549, Calu-6, Calu-3) in vitro using a retrovirus vector. The transfected lung cancer cell lines secreted 22-73 ng TNF/ 10^6 cells/24 hr whereas the non-transfected parental cells produced none. There was no difference in in vitro cell growth between TNF cDNA-modified cells and parental cells and all cell lines were insensitive to the direct effect of TNF. The in vivo tumorigenicity of the parental and modified cells were compared in nude mice, animals that permit the growth of human tumors but

can respond to TNF. Mice injected subcutaneously with parental cells all developed tumors (32/32 tumors by 4 wk). Strikingly, there was markedly less tumor growth with TNF cDNA-modified cells (6/32 tumors developed by 6 mos). Importantly, the administration of anti-TNF antibodies at the site of injection of modified cells permitted tumor development, confirming the role of tumor cell TNF production in suppressing tumorigenicity. Mice receiving a mixture of 50% modified and 50% parental cells had decreased tumor growth in all cell lines, demonstrating that the modified cells could suppress the tumorigenicity of parental cells. These data suggest that TNF can induce host defense processes to suppress in vivo growth of human tumor cells and provide a rationale for transfer of the human TNF cDNA to malignant cells for the therapy of lung cancer.

Based on the in vivo efficacy and safety data developed in the Pulmonary Branch over the past 2 years regarding the use of an El-E3⁻ replicator deficient recombinant adenovirus vector to transfer the normal human CFTR cDNA to the respiratory epithelium in vivo in experimental animals, we developed a protocol to evaluate this vector for in vivo gene therapy of the respiratory manifestations of CF. The protocol was approved by the NIH Recombinant DNA Advisory Committee on December 4, 1992 and by the Food and Drug Administration on April 16, 1993. On April 17, 1993 we carried out the first human study for gene therapy of CF, with the administration of 2×10^5 pfu of the AdCFTR vector to the nasal epithelium. On the next day, 2×10^7 pfu were administered to the lung. To date, 3 patients have been evaluated. The data is currently being analyzed for safety and efficacy parameters relevant to the recombinant vector.

II. Lung inflammation

A large number of acute and chronic lung disorders are characterized by chronic inflammation in the lung. The list of these disorders includes, CF, α 1AT deficiency, cigarette smoking induced bronchitis and emphysema, interstitial lung disease, such as asthma and adult respiratory distress syndrome. In the past year, studies in the Pulmonary Branch have focused on inflammatory mediators, defenses against inflammation, and therapeutic strategies to prevent protease and oxidant damage.

Catalase, a 240 kDa cytoplasmic protein, catalyzes the breakdown of H_2O_2 into H_2O and O_2 . Evaluation of freshly isolated human airway epithelial cells from normal individuals ($n=24$) demonstrated that catalase mRNA levels are very low (1.3 ± 0.2 mRNA copies/cell), suggesting that if catalase is to play an important role in protecting the airway epithelium, catalase mRNA levels would have to be upregulated in periods of oxidant stress. To examine this concept, catalase expression was quantified in the human bronchial epithelial derived cell lines, BET-1A and HS-24. As in freshly isolated airway cells, catalase mRNA levels in both cell lines were low. Importantly, catalase mRNA expression in both cell lines was not up-regulated by oxidant stress, including glucose oxidase (50 mU/ml, 24 hr), menadione (100 μ M, 8 hr), H_2O_2 (400 μ M, 24 hr), or 85% oxygen (24 hr). To examine the mechanisms underlying this lack of response to oxidants, 1.7 kb of the 5'-flanking region of the catalase gene was analyzed. There was no TATA promoter element, but a high G + C content, four CCAAT boxes, and two

Spl binding sites. Primer extension analysis identified multiple transcription start sites located between 61 bp and 235 bp upstream from the translation start codon. Transfection studies with HS-24 cells using fusion genes composed of fragments with various lengths of the 5'-flanking region (up to 1.7 kb) of the catalase gene and a luciferase reporter gene revealed no strong promoter activity (0.9 to 2.7% of a control Rous sarcoma virus promoter). Further, the pattern of luciferase activities of HS-24 cells transfected with these fusion genes was not changed after exposure of transfected HS-24 cells to 85% oxygen for 24 hr. Together, the structural and transcriptional features suggest that the catalase gene promoter has characteristics of a "housekeeping" gene, a feature typical of genes coding for other peroxisomal enzymes. The lack of response of catalase gene expression to various oxidants in human bronchial epithelial cells likely results from these characteristics of the 5'-flanking region of the catalase gene, and helps to explain why bronchial epithelial cells are so vulnerable to oxidant injury.

Phospholipase A₂ (PLA₂) is the rate limiting enzyme in the production of arachidonic acid, the substrate for the formation of prostaglandin and leukotriene mediators of inflammation. There are two forms of PLA₂, a 14 kDa secretory (sPLA₂) enzyme, and an 85 kDa cytosolic (cPLA₂) enzyme. To begin to characterize the importance of the genes coding for these enzymes in cells relevant to potential inflammatory processes in the lung, we evaluated mRNA transcripts of sPLA₂ and cPLA₂ in several human tissues and cell lines in comparison to lung tissue, bronchial epithelium and alveolar macrophages. The expected 0.8 kb mRNA transcripts for sPLA₂ were detected at very low levels in lung tissue, airway epithelium and alveolar macrophages, but at levels far less than in liver, heart and placenta. In contrast, the 2.8 kb cPLA₂ mRNA transcripts were present at moderate to high levels in a broad variety of tissues including lung tissue, airway epithelium and alveolar macrophages, i.e., the genes for both the secretory and cytosolic form of PLA₂ are expressed in cells of the airway epithelial surface, but expression of the cytosolic form dominates. To evaluate the regulation of these genes, we determined the structure of the promoter of the cPLA₂ gene, and examined the effects of inflammatory cytokines on mRNA transcript levels of both genes on human airway epithelial (BET-1A), macrophage (U937) and hepatocyte (HepG2) cell lines. Sequence analysis of the cPLA₂ gene promoter demonstrated features of a "housekeeping" promoter, suggesting that it is expressed in a constitutive fashion, markedly different from the known structure of the sPLA₂ promoter which has features suggesting it can be regulated by exogenous signals. Consistent with this analysis, various cytokines (TNF, IL-1 and IL-6, all 1000 u/ml, 24 hr) had no effect on cPLA₂ gene expression in BET-1A cells, but in contrast markedly upregulated sPLA₂ gene expression in HepG2 cells. Thus, while both the secretory and cytosolic PLA₂ genes are expressed in lung, the cPLA₂ gene dominates and its expression appears to be constitutive, suggesting that regulation of this enzyme system on the airway epithelial surface may be at the protein rather than the mRNA level.

Secretory leukoprotease inhibitor (SLPI), a 12 kDa antiprotease, serves as the major neutrophil elastase (NE) inhibitor on the epithelial surface of the upper respiratory tract. In diseases such as cystic fibrosis (CF),

which are characterized by a marked neutrophil-dominated inflammation on the respiratory epithelial surface, the anti-NE defenses of the respiratory tract are overwhelmed by a large NE burden, with resultant chronic lung damage. To evaluate the ability of aerosolized recombinant SLPI (rSLPI) to augment anti-NE defenses on the respiratory epithelial surface, rSLPI was aerosolized as a single 100 mg dose to 12 normal volunteers (24 ± 2 yr). Following rSLPI aerosolization, SLPI levels in epithelial lining fluid (ELF) rose to $16.1 \pm 0.3 \mu\text{M}$ ($n=3$) at 1 hr, and to $8.4 \pm 1.5 \mu\text{M}$ ($n=3$) at 4 hr, $4.9 \pm 2 \mu\text{M}$ ($n=3$) at 8 hr and $3.9 \pm 1.4 \mu\text{M}$ ($n=3$) at 12 hr. All values were significantly greater ($p<0.02$) than baseline values for normals ($1.1 \pm 0.3 \mu\text{M}$). The anti-NE capacity in ELF followed a similar pattern with levels of $17.1 \pm 0.7 \mu\text{M}$, $7.8 \pm 2.0 \mu\text{M}$, $6.4 \pm 2.0 \mu\text{M}$ and $3.6 \pm 0.9 \mu\text{M}$ at the same time points; all values were significantly greater than values for normals ($1.9 \pm 0.2 \mu\text{M}$, $p<0.05$). To determine the ability of rSLPI to suppress NE *in vivo*, rSLPI was aerosolized to 24 individuals with CF (30 ± 4 yr) at a dose of 100 mg twice daily for 1 wk ($n=16$) or 50 mg twice daily for 2 wk ($n=8$). The pre-therapy levels of SLPI in ELF in the CF individuals was the same as in normals, ($0.7 \pm 0.1 \mu\text{M}$, $p>0.6$) but, unlike normals, individuals with CF had active NE in ELF ($12.4 \pm 3 \mu\text{M}$). The ELF SLPI levels evaluated 12 hrs after 1 wk of the 50 mg, twice daily rSLPI dose regime ($3.0 \pm 0.7 \mu\text{M}$) were similar to the levels at 2 wks ($3.4 \pm 0.6 \mu\text{M}$, $p>0.6$) but were insufficient to significantly decrease ELF active NE levels. However following aerosolization of 100 mg rSLPI twice daily for 1 wk, SLPI ELF levels rose ($6.2 \pm 0.8 \mu\text{M}$) with concomitant reduction in ELF NE ($6.1 \pm 1.2 \mu\text{M}$, $p<0.05$). These data suggest that rSLPI is functional following *in vivo* aerosolization and can significantly suppress the NE burden in the CF lung but must be given regularly and in sufficient quantity to have this effect.

The gene coding for the airway epithelial cell specific Clara cell 10 kDa protein (CC10) is expressed at high level in human bronchial epithelium, where it likely functions as an inhibitor of inflammatory processes. To begin to evaluate the regulation of this gene in the airways, we characterized the structure and location of the CC10 gene. Following localization of the human gene to chromosome 11 by screening genomic DNA from human-hamster hybrid cell lines by the polymerase chain reaction (PCR) using human CC10 specific primers, the gene was cloned from a human chromosome 11 specific genomic DNA library. Sequence analysis demonstrated a 4.1 kb gene with 3 exons, a long (3 kb) first and short (0.65 kb) second intron, and 4 Alu repeat sequences within the introns. Screening of Southern blots of human genomic DNA for restriction fragment length polymorphisms and screening the products of PCR amplification of genomic DNA from 84 individuals by gel electrophoresis demonstrated a polymorphism of the CC10 gene with an allelic frequency of 3.0%. Observation of the inheritance of this polymorphism within the gene in a 3 generation family in comparison to other polymorphic chromosomal markers permitted subchromosomal localization of the human gene to between markers D11S97 and D11S16 on chromosome 11. Interestingly, sequence analysis demonstrated that the polymorphism resulted from a human-specific Alu repeat inserted into the second intron, 45 bp from the second exon-intron junction. However, this insertion did not induce changes in mRNA transcripts, as has been described for polymorphic Alu insertions in other genes. In this regard, PCR analysis of full length mRNA obtained from the proximal bronchial epithelium of 78 individuals (50 normal, 18 inter-

stitial lung disease, 5 chronic bronchitis, 5 emphysema) revealed no evidence of alternatively spliced forms or gene rearrangements resulting from recombination between the frequent Alu repeats. Based on the anti-inflammatory functions of the CCL10 protein, the structure, localization and expression of the CCL10 gene may have a significant impact on modulating airway inflammation.

Interleukin-8 (IL-8), an 8.5 kDa cytokine which attracts and activates neutrophils, can be produced by a variety of lung cells, including fibroblasts, endothelial cells and epithelial cells. There is growing evidence that the exaggerated expression of the IL-8 gene by lung cells plays an important role in the cascade of pathogenic processes in disorders characterized by neutrophil-dominated respiratory epithelial inflammation such as cystic fibrosis, idiopathic pulmonary fibrosis and adult respiratory distress syndrome. In this regard, and with the knowledge that respiratory epithelial cells are capable of expressing the IL-8 gene, we have developed an animal model of neutrophil-dominated lung inflammation by the novel strategy of using a replication deficient recombinant adenovirus containing the human IL-8 cDNA driven by the cytomegalovirus major immediate early promoter (AdCMVIL-8) to transfer the IL-8 cDNA to the respiratory epithelium, and thus express human IL-8 in the local milieu. When compared with control adenovirus-infected mice, lung lavage analysis of mice 3 d after receiving 3×10^9 pfu of AdCMVIL-8 intratracheally showed human IL-8 (control 0 ng/ml; AdCMVIL-8 32 ± 4 ng/ml). Consistent with this observation, at the same time there was elevation of the percentage of neutrophils in lavage fluid (control $12 \pm 5\%$, AdCMVIL-8 $75 \pm 2\%$, $p < 0.001$) and of total neutrophil number recovered in lavage fluid [control 2 ± 1 ($\times 10^4$), AdCMVIL-8 71 ± 12 ($\times 10^4$), $p < 0.005$]. Histological examination of the lungs demonstrated that while the control adenovirus-infected mice had only mild inflammation mainly comprised of mononuclear cells, the AdCMVIL-8 infected mice showed moderate to severe neutrophil-dominated inflammation of bronchioles and alveoli. This study represents the successful demonstration of *in vivo* gene transfer to establish an animal model of neutrophil-dominated lung inflammation and is consistent with the concept that IL-8 can play an important role in lung injury.

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